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Dose Response and Functional Consequences of Choline Induced Changes in Carnitine Homeostasis in Guinea Pigs

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To the Graduate Council:

I am submitting herewith a dissertation written by James William Daily entitled "Dose Response and Functional Consequences of Choline Induced Changes in Carnitine Homeostasis in Guinea Pigs." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Dileep S. Sachan, Major Professor

We have read this dissertation and recommend its acceptance:

Jay Whelan, Michael Zemel, Robert Moore

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

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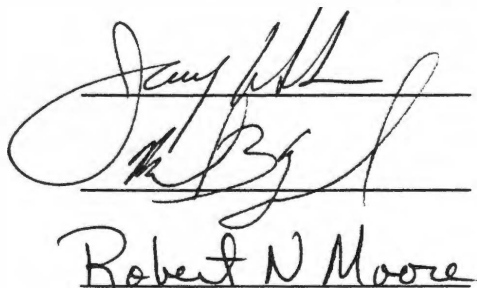
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We have read this dissertation
and recommend its acceptance:



Robert N Moore

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

**Dose Response and Functional Consequences
of Choline Induced Changes in Carnitine
Homeostasis in Guinea Pigs**

**A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

**James William Daily III
August 1996**

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ABSTRACT

This study further characterized a newly discovered interaction of two nutrients, choline and carnitine. Increased dietary choline had previously been demonstrated to cause a conservation of carnitine in humans and guinea pigs, and an increased muscle carnitine concentration guinea pigs. The first part of this study compared the effect of varied doses of choline on urinary excretion of carnitine, tissue and whole body carnitine concentrations, and body composition. It was demonstrated that a choline dose of 2 g/kg diet results in a near maximal effect on urinary conservation of carnitine, which is mediated by an increase in the fractional tubular reabsorption of carnitine. There was also a dose dependent decrease in the ratio of fat/protein of carcasses of choline supplemented animals. The second part of the study evaluated functional consequences of the choline-carnitine interaction. Indirect calorimetry was used to evaluate energy substrate utilization in choline supplemented (3g/kg diet) and nonsupplemented guinea pigs. There was no effect of choline supplementation on fatty acid oxidation in the guinea pigs when fed, unfed, or during treadmill exercise. The mechanism of how choline supplementation affects body fat composition in guinea pigs remains unknown, but appears to be independent of effects on capacity for fatty acid oxidation.

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Chapter 1

Introduction

Two molecules are of primary importance in the research presented here: choline, and carnitine. Choline and carnitine are both common constituents of most diets, but carnitine is not an essential nutrient and choline is not universally recognized as essential (Rebouche 1986 and Zeisel et al. 1991).

We have previously shown that choline supplementation, results in a substantial decrease in the urinary excretion of carnitine in humans (Daily and Sachan 1995, Dodson and Sachan 1996). The decreased excretion of carnitine was accompanied by variable changes in plasma carnitine concentrations, and it was concluded that there was no significant effect on plasma carnitine concentrations. In rats, carnitine excretion was unaffected by choline both in our studies and those of others (Daily and Sachan 1995, Sheard et al. 1992). Guinea pigs, however, did exhibit a pattern of carnitine excretion similar to that of humans during choline supplementation, but had an increased plasma carnitine concentration, unlike humans. We have attributed the lack of effect in rats to the high choline oxidase activity in rat liver, 60 fold higher than that in humans and 20 fold higher than that guinea pigs (Sidransky & Farber 1960).

There are several possible explanations for the decreased excretion of carnitine during choline supplementation. Absorption of dietary carnitine could be impaired and less carnitine available to be excreted. The guinea pig diet, however, did not have enough carnitine to be detectable and should not have been able to contribute enough carnitine to be significant. Choline may have impaired the biosynthesis of carnitine, but that seems unlikely because choline deficiency results in a carnitine deficiency (Carter & Frenkel 1978) and excess has not been shown to impair synthesis. Another likely explanation is that carnitine is being redistributed among body pools with some tissues taking up carnitine. If this were the case, it might indicate an increased need for carnitine in the affected tissues and be an indicator of increased fatty acid oxidation or at least an increased capacity for fatty acid oxidation.

Choline and carnitine are all sold as supplements in the United States. Any interaction between them that might affect the status of the other would have significant implications. We have demonstrated that choline does affect carnitine homeostasis. In guinea pigs this has been demonstrated at a level of 3g/kg diet of supplemental choline. The first study presented here is a dose response study to determine what levels of supplementation are required for a maximal effect of choline on urinary carnitine excretion and carnitine concentrations in guinea pig plasma and tissues. Because of the obligatory role of carnitine in the oxidation of fatty acids, the second study investigates the physiological consequences of choline supplementation on fat metabolism in guinea pigs.

Chapter 2

Literature Review

Choline

Choline is a quaternary trimethylated amine as is carnitine, another nutrient of primary importance in this research. Choline is synthesized de novo in humans, but is also widely available in the diet. Choline deficiency has been induced in animals resulting in liver damage, renal hemorrhage, and inability to export triglyceride from the liver, thereby causing hepatic fat accumulation (Kuksis & Mookerjea 1984). There has never been a case of choline deficiency syndrome observed in humans, probably because of its ubiquitous nature in the human diet.

Choline in the diet:

Daily dietary intake of choline is estimated at between 600 and 900 mg/day (Kuksis A. & Mookerjea 1984). Choline is available from dietary sources both as choline chloride and as phosphatidylcholine (lecithin). The best dietary sources of choline include meat, eggs, oatmeal, soybeans, wheat germ, peanuts, and other nuts and grains (Zeisel 1981). The most abundant form of choline in the diet is lecithin, but many foods contain significant amounts of choline chloride, especially liver and soybeans. Choline is also obtained in the diet from sources other than naturally

occurring foods. Lecithin is a common food additive because of its ability to emulsify fats. It is a major ingredient in frying pan sprays, and is available as a nutritional supplement as either lecithin or choline chloride.

Non dietary sources of choline:

Lecithin is formed in mammals by three different pathways; the base exchange pathway, CDP-choline pathway, and the methyltransferase pathway (Zeisel 1981). The base exchange pathway simply exchanges choline for another base on a phospholipid. The CDP-choline pathway uses diacylglycerol and pre-existing choline to form phosphatidylcholine. The methyltransferase pathway is the only way that new choline is produced. In the methyltransferase pathway phosphatidylethanolamine is sequentially methylated by phosphatidylethanolamine-N-methyltransferase (PEMT) using S-adenosyl methionine (SAM) as the methyl source (Bjoranstad P. & Bremer J. 1966 and Lindblad L. & Schersten T. 1976). PEMT is most concentrated in the liver but is also found in the kidney, testes, heart, lung, adrenal gland, erythrocytes, spleen, and brain (Blusztajn J.K. et al. 1979, Hirata & Axelrod 1978, and Hirata et al. 1978). In the rat about 15% of the choline requirement is supplied by the methyltransferase pathway, and the remainder obtained from the diet (Zeisel 1981, Scheneider & Vance 1979, and Wise & Eley 1965). Human requirements for dietary choline, if any, are not yet determined.

Choline in single carbon metabolism:

Choline is frequently referred to as an important source of labile methyl groups (Zeisel & Da Costa 1991). SAM is the active agent in many synthesis reactions requiring the addition of a methyl group. When SAM donates its methyl group it is hydrolyzed to form homocysteine and adenosine. The methionine may be regenerated by the transfer of a methyl group from either N⁵-methyl tetrahydrofolate or betaine. Both sources are of about equal importance in the rat (Finkelstein & Martin 1984). Choline supplementation in rats has been shown to prevent pathology of folate deficiency for up to three months (Aarsaether et al. 1988). Although choline is a significant contributor of methyl groups in rats, its significance in humans is questionable. The only way for choline to be used as a methyl source is for it to first be oxidized to betaine. Human liver has only 1/60 of the choline oxidase activity of rat liver (microliters of O₂ uptake/hr/g wet liver) (Sidransky & Farber 1960). All studies of choline as a methyl donor have used rats as an animal model and may not apply to humans. However, even if humans do not utilize choline as a methyl donor, there are other critical functions for choline in humans.

Functions of choline in humans:

The central nervous system requires choline for the synthesis of the important neurotransmitter, acetylcholine. Acetylcholine is important for communication between neurons in the central nervous system (CNS) and is also the neurotransmitter

used for communication between the CNS and somatic motor neurons. Acetylcholine is synthesized by the transfer of an acetyl group from acetyl coenzyme-A (CoA) to choline in a reaction catalyzed by the enzyme choline acetyltransferase. Enzyme kinetic studies of choline acetyltransferase in brain tissue suggest that the enzyme is unlikely to ever be saturated, and that the rate of acetylcholine synthesis is controlled by the availability of choline and/or acetyl CoA (White and Wu 1973). It has been demonstrated that administration of choline simultaneously with an inducer of cholinergic activity (3,4-diaminopyridine) increases the synthesis and release of acetylcholine in rats and that the two act synergistically for the greatest effect (Buyukuysal, R.L. et. al. 1995).

Choline, as phosphatidylcholine and sphingomyelin, is a major structural component of cell membranes. Phosphatidylcholine accounts for approximately 50% of all cell membrane phospholipids and sphingomyelin 5-20% depending on the cell type (Zeisel 1993).

Phosphatidylcholine is an important source of diacylglycerol used in maintaining active protein kinase C within cells. The activation of protein kinase C is mediated by binding to calcium and diacylglycerol in most isotypes, but some require only diacylglycerol (Nishizuka 1992 and Exton 1990). Choline is also a part of the membrane phospholipid, sphingomyelin, which is involved in intracellular signaling. Sphingomyelin is hydrolyzed to form ceramide and choline phosphate in response to several known factors including 1- α ,25-dihydroxyvitamin D, tumor necrosis factor,

and γ -interferon (Merril 1992). Ceramide is a potent inhibitor of cell growth and sphingosine an inhibitor of protein kinase C (Merril & Stevens 1989 and Merrill & Jones 1990). Choline itself has not yet been reported to be a signal transduction molecule and its function, if any, when choline containing phospholipids are hydrolyzed, is unknown.

Choline, an essential nutrient:

Choline has been shown to be an essential nutrient in many species including rat, hamster, guinea pig, pig, dog, monkey, trout, and chicken (Finkelstein et al. 1982). Choline deficiency results in spontaneous liver cancer in rats without exposure to known carcinogens and is the only nutrient whose deficiency is known to result in spontaneous malignancies (Newberne & Roger 1990, and Zeisel 1993). Choline deficiency, in species for which it has been shown to be essential, can result in impaired hepatic, renal, and pancreatic function; memory loss, and decreased growth (Finkelstein et al. 1982). It has been difficult to demonstrate that choline is an essential nutrient for humans; however, it has been shown that human cells grown in culture have an absolute requirement for choline (Eagle 1955). Malnourished humans have decreased serum choline concentrations and parenterally fed persons without choline supplementation develop abnormal liver function, indicating that there may be a requirement for choline in the diet (Tayek et. al. 1990).

Zeisel et al. (1991) conducted a human study in which the experimental group received an artificial diet free of choline and a control group which received an identical diet, except with added choline. They found decreased choline and phosphatidylcholine in the plasma as expected, but also, the serum alanine aminotransferase activity increased consistently over the three weeks in the choline deficient group, and the study was terminated to avoid risk to the subjects.

A common problem among patients on long-term total parental nutrition (TPN) is hepatic steatosis accompanied by lower than normal plasma choline concentrations (Buchman et. al. 1992, Buchman et. al. 1993). A study of 4 patients receiving long-term TPN (9.7 ± 4.7 years) who had low plasma choline concentrations as well as hepatic steatosis, evaluated choline supplementation as a treatment for the steatosis (Buchman et. al. 1995). The degree of hepatic steatosis was determined by the use of abdominal computed tomography (CT) before beginning the choline supplementation, and after 2 and 6 weeks of supplementation. Liver density, in Hounsfield units (HU) can be used to estimate the degree of steatosis, since fat is less dense than lean tissue. Liver density in the patients increased from -14.2 ± 22.3 HU at the beginning of the study to 8.4 ± 10.3 HU after 2 weeks, and 9.6 ± 10.7 HU after 6 weeks. After 6 weeks of choline supplementation, there was essentially no hepatic steatosis in the patients.

Pregnancy and lactation are also conditions that may be associated with compromised choline status. A choline deficient diet in both pregnant and lactating

rats results in significant decreases in liver phosphorylcholine, betaine, and free choline as compared to non-pregnant/non-lactating rats on a choline deficient diet (Zeisel, S.H, et. al. 1995). This demonstrates that pregnancy and lactation result in increased risk for choline deficiency in rats. However, this has yet to be demonstrated in humans.

Impairment of liver function seems to be consistent among all species, including humans, during choline deficiency. It has been difficult to find cases of choline deficiency in human populations because choline is plentiful in foods. It would be difficult to construct a diet of natural foods that is choline deficient, this does not mean, however, that choline is not essential - just easily obtained. Studies with artificial diets suggest that choline may be an essential nutrient.

Carnitine

L-carnitine, L- β -hydroxy- γ -N-trimethylaminobutyric acid, like choline is a quaternary, trimethylated amine. Carnitine, again like choline, is synthesized de novo in humans, and is available in the diet, though not as plentifully as choline (Broquist 1994). Carnitine was originally called vitamin B_T but this term is no longer used since carnitine is not considered essential in the diets of higher organisms. Diseases of carnitine deficiency have been identified in humans (Broquist 1994), however, and will be discussed later.

Carnitine in the diet:

Dietary carnitine is derived primarily from animal food sources, and the average nonvegetarian diet is estimated to supply 100-300 mg of carnitine per day (Broquist 1994). Vegetables, fruits, and grains are very poor sources of carnitine. Ground beef has about 580 μmol of carnitine per 100 grams whereas asparagus (one of the richest vegetable sources) has about 1.2 μmol per 100 grams (Broquist 1994, Moukarazel et al. 1992).

Dietary intake of carnitine has been shown to have a significant influence on carnitine status in humans. Children receiving total parenteral nutrition for 7.2 ± 2.6 years from birth had total plasma carnitine concentrations approximately half that of other children of similar age (Moukarazel et al. 1992). When adults and children eat either strict vegetarian diets, lacto-ovovegetarian diets, or mixed diets (including meat) for long periods of time, both plasma carnitine and urinary excretion of carnitine have been shown to be affected (Lombard et al. 1989). Differences in plasma carnitine concentrations between groups were small but significantly lower in vegetarians. Differences in urinary excretion of carnitine were much greater, adult men on mixed diets, lactoovovegetarian diets, or strict vegetarian diets excreted 5.79 ± 3.08 , 2.1 ± 0.76 , and 1.36 ± 0.49 μmol carnitine per kg body weight per day, respectively. Similar trends were seen for male children as well as female adults and children. The urinary excretion of carnitine decreased proportionately with predicted carnitine intake. The

subjects eating diets highest in carnitine had the highest excretion rate and those with diets lowest in carnitine (strict vegetarian) had the lowest excretion rate.

How the differences in plasma carnitine and urinary carnitine excretion correlate with tissue concentrations is not certain. It has been shown in rats that dietary differences do substantially affect tissue concentrations, but were roughly proportional to plasma concentrations, which had a much greater variation than seen in the above mentioned human study (Negaro et al. 1987).

To what extent humans rely on dietary carnitine is unknown. Cederblad and Lindsted (1976) report that synthesis contributes four times more carnitine than does diet in laboratory rats. In humans, however, dietary intake may be more important since diet typically contributes 100-300 mg per day. The functional consequences of variations in dietary intake of carnitine in humans are not known at this time, but it is clear that dietary intake can affect plasma carnitine concentrations in humans and both plasma and tissue concentrations in rats.

Intestinal absorption and tissue uptake of carnitine:

It has been difficult for researchers to determine the percentage of carnitine absorbed from the diet. It was assumed for many years that carnitine was nearly 100% absorbed because when labeled carnitine was given orally, little radioactivity appeared in the feces. Kinetic analysis in animals and humans, however, indicated that there was a higher rate of excretion than was reported (Brooks & McIntosh 1975, Rebouche &

Chenard 1991, Rebouche & Engel 1983). In the kinetic studies, the carnitine was given intravenously and no metabolites of carnitine were found in urine or feces. However, when radioactively labeled carnitine was fed orally to rats as much as 34% of the radioactivity was found in excreted metabolites (trimethylamine N-oxide and γ -butyrobetaine) of carnitine in urine and, to a small extent, in feces (Rebouche et al. 1984). When humans are fed radioactive carnitine 95% of the radioactivity is absorbed from the intestinal tract, but several metabolites show up in the urine, primarily trimethylamine N-oxide (Rebouche & Chenard 1991). Apparently intestinal bacteria degrade carnitine to other metabolites which are in turn absorbed and then excreted in the urine. Recent studies indicate that carnitine is 54-87% absorbed without degradation, depending on the dosage (Rebouche & Chenard 1991).

Carnitine is absorbed both actively and passively across the intestinal mucosa (Broquist 1994). Active carnitine absorption is a sodium-linked process similar to the absorption of phosphate (Cheng & Sacktor 1981, Hamilton et al 1983, Stadler et al. 1993.). Carnitine enters the blood stream via the portal circulation and circulates as free or acyl carnitines (Broquist 1994). Most tissue cells transport carnitine against a concentration gradient resulting in a 10-100 fold greater intracellular concentration (Broquist 1994), suggesting an active transport mechanism.

Carnitine biosynthesis:

Carnitine is synthesized from two essential amino acids in all mammals including humans (Bieber 1988). The nitrogen and carbon backbone of carnitine is derived from lysine, and the methyl groups attached to the nitrogen originate from SAM. **Table 2-1** shows the steps in carnitine biosynthesis, and the nutrients required as cofactors (Broquist 1994, Cederblad et al. 1979, Henderson et al 1982, Lindstedt et al 1970, Hulse and Henderson 1980).

Table 2-1
Steps in the Biosynthesis of Carnitine, and Nutrients Required

Step	Reactant	Product	Required Nutrient
1.	Lysine	N-trimethyl-lysine	Methionine, Lysine
2.	N-trimethyl-lysine	N-trimethyl-3-hydroxylysine	Vitamin C, Iron
3.	N-trimethyl-3-hydroxylysine	4-N-trimethylamino butyraldehyde	Vitamin B6
4.	4-N-trimethylamino butyraldehyde	4-N-trimethylamino butyric acid	Niacin
5.	4-N-trimethylamino butyric acid	L-carnitine	Vitamin C, Iron

In step 1 lysine is methylated as a post-translational modification in proteins including actin, myosin, and histones; and N-trimethyl-lysine is released as a result of enzymatic degradation of those proteins (Broquist 1994, Broquist 1982, LdaBadie et al. 1976). Steps 2-5 in Table 2-1 occur in both the liver and kidney in higher mammals (Broquist 1982, Sachan & Hoppel 1980, Rebouche and Engel 1980).

It is not clear how much effect diets deficient in the nutrients involved in carnitine biosynthesis have on carnitine status. Wheat gluten diets (deficient in lysine) have little effect on carnitine concentrations in adult rats (Sachan & Mynatt 1993), however, weanling rats fed a lysine deficient diet will become carnitine deficient (Khan & Bamji 1979, Tanphaichitr & Broquist 1973).

Vitamin C deficiency has also been proposed to impair carnitine status since it is required for its biosynthesis. Vitamin C deficiency has been shown to result in decreased activity of hepatic γ -butyrobetaine hydroxylase and hepatic and renal ϵ -N-trimethyllysine hydroxylase (Rebouche 1991). It was not determined, however, if the effect on enzyme activity altered carnitine status. A subsequent study (Rebouche 1995) demonstrated that tissue carnitine concentrations were reduced in scorbutic guinea pigs, and that urinary excretion of carnitine was increased. However, supplements of radioactive tracers of the carnitine precursor, ϵ -N-trimethyllysine, were converted to carnitine more rapidly in the scorbutic guinea pigs than in controls. It was concluded, therefore, that vitamin C deficiency causes carnitine depletion by increasing urinary excretion of carnitine and not by inhibiting its synthesis.

Iron also has an obligatory role in carnitine synthesis and a deficiency could impair the process. Nutritional iron deficiency in pregnant rats results in hypertriglyceridemia in both mother and pups, which can be reversed with iron supplementation (Sherman et al. 1982). Pregnant rats continued on a iron deficient diet into the lactation period produce milk with normal carnitine concentrations but reduced iron concentrations (Bartholemy & Sherman 1985). On day 2 after birth there was no difference in carnitine concentrations in the pups, but on day 16 liver carnitine was about half that of iron sufficient pups. Rat pups are not fully able to synthesize carnitine until 8 days after birth (Hahn 1981), so the effect of iron deficiency on their own carnitine synthesis would not be expected to be seen very soon after birth.

Marzo et al. (1994) found that plasma concentrations and urinary excretion of carnitine progressively decreased significantly in pregnant women. Plasma concentrations dropped to approximately half the normal values for the same subjects by week 36 of the pregnancy. The decreased plasma carnitine concentrations were restored without intervention by one month after giving birth. These studies demonstrate that iron deficiency may result in impaired carnitine synthesis and may also explain the hypertriglyceridemia seen in the iron deficient pups.

Carnitine and fatty acid metabolism:

The transport of long chain fatty acids across the inner mitochondrial membrane into the matrix of the mitochondria (**Figure 2-1**) is the best characterized function of carnitine (Fritz 1963). This process involves the activation of a fatty acid by coupling to coenzyme A via a thioester bond, followed by transfer of the acyl group to carnitine, translocation of the acylcarnitine across the inner membrane, and transfer of the acyl group to intramitochondrial CoA (Beiber 1988). The first step in carnitine mediated transport of fatty acids across the inner mitochondrial membrane is the cytosolic generation of acyl CoA which diffuses across the outer membrane into the inter membrane space.

Carnitine palmitoyltransferase I (CPT1) spans the outer mitochondrial membrane, with the transferase active site on the inside of the membrane and a malonyl CoA binding site on the cytosolic side. The active site transfers the acyl group of the acyl-CoA to carnitine to make an acylcarnitine (Bremer 1983). Binding of malonyl CoA to the cytosolic side of the CPT1 inhibits the enzyme and assures that acyl groups will not be transported into the mitochondria for oxidation at the same time that fats are being synthesized (Brady et al 1993). Carnitine-acylcarnitine translocase shuttles the acylcarnitine across the inner mitochondrial membrane where another CPT (CPT₂) transfers the acyl group back to intramitochondrial CoA after which it proceeds to oxidation (Broquist 1994). The reverse of the process for transporting long chain

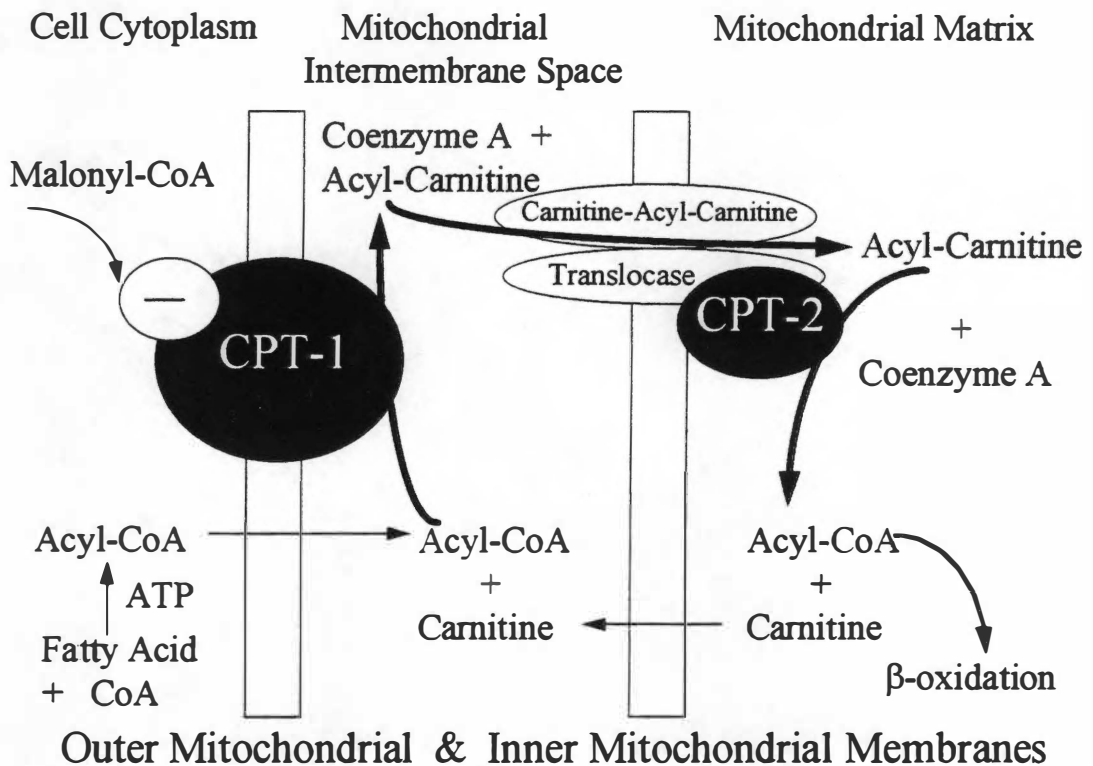


Figure 2-1: Role of carnitine in the translocation of acyl groups across the inner mitochondrial membrane into the mitochondrial matrix for oxidation.

fatty acids into the mitochondrial matrix appears to be used for removing short and medium chain fatty acids from the inside of the mitochondria (Beiber 1988, Broquist 1994). This function of removing short-chain fatty acids from the mitochondria may be related to the observed increases in acid-soluble acyl carnitine (ASAC), short-chain acyl carnitine, in serum and urine of humans and rats during fasting (Frohlich et al 1978 and Seecombe et al 1978). Free, or non-esterified, carnitine (NEC) is decreased in serum during fasting and is inversely correlated with both serum free fatty acid and β -hydroxybutyrate concentrations (Frohlich et al 1978). Increased ASAC, with a concomitant decrease in NEC; during fasting, suggests that increased fatty acid oxidation may result in a shift in body pools from non-esterified to esterified carnitine. The correlation of ASAC and inverse correlation of NEC with serum free fatty acids and β -hydroxybutyrate suggests that increases in the ratio of ASAC/NEC are indicative of an increase in the oxidation of fatty acids.

Other possible functions of carnitine:

The concentration of carnitine in the brain is lower than most tissues, but still significant (Rebouche 1986). The function of carnitine in the brain is unknown since the brain is not believed to utilize fatty acids for energy. Recent studies have investigated the use of acetylcarnitine for treatment of neurological disorders. In streptozotocin induced diabetic rats, treatment with acetylcarnitine was able to reverse neurological damage as determined by measurement of nerve conductance velocity

(Morabito et al. 1993). In aged rats, acetylcarnitine was able to restore neuromuscular conduction velocity to that of young rats after 6 months of treatment (Scarfo et al. 1992). Human tests with Alzheimer's disease patients have shown some evidence that acetylcarnitine may ameliorate the symptoms of the disease when given orally (Sano et al. 1992). How acetylcarnitine may exert the effects reported on neurological function is unknown.

The organ most sensitive to carnitine deficiency is the heart, because the heart relies on fatty acids for about 60% of its energy needs (Rebouche 1986). Primary carnitine deficiency is a rare syndrome that has been described in children (Pierpont 1992). In this disease plasma carnitine concentrations are very low resulting in congestive cardiomegaly which can be reversed by oral carnitine administration. Cardiomegaly can also develop as a result of carnitine being dialyzed out of the blood in hemodialysis patients, and carnitine should be supplemented in such patients (Kudoh et al. 1983). The use of carnitine has been investigated for treatment of ischemic heart disease, but the results thus far have been contradictory so it is still under investigation (Rebouche 1986).

Carnitine has also been shown to reduce the hepatic steatosis seen in chronic alcoholic rats (Sachan & Rhew 1983). Rats fed a diet with 36% of the calories from alcohol will accumulate 3-4 times the hepatic lipid content of controls within 8 weeks. Rats supplemented with D,L-carnitine (1% by weight in the diet or 0.5% L-carnitine) accumulated less than half the lipids as did the non-supplemented alcoholic rats. The

effect of carnitine on liver lipids was later shown to be dose dependent with the greatest effect at 0.8% D,L-carnitine in the diet (Rhew & Sachan 1986). Carnitine was also shown to reduce the rate of alcohol disappearance from the blood (Sachan & Berger 1993). The effect was not seen with choline supplementation although choline is very similar in structure to carnitine (Sachan & Berger 1993). It was concluded that the attenuation of ethanol metabolism was specific for carnitine. The reduced rate of ethanol clearance was shown to be the result of reduced ethanol oxidation, even though the activity of ethanol oxidizing enzymes was unaffected (Mynatt & Sachan 1992).

The hepatic steatosis resulting from aflatoxin B₁ or CCl₄ administration has also been shown to be reduced by carnitine supplementation (Sachan et al. 1993, Sachan and Dodson 1992, Yatim and Sachan 1992). Carnitine also reduced formation of aflatoxin adducts with RNA, DNA, and proteins in the liver (Yatim and Sachan 1992).

Carnitine excretion:

At normal plasma concentrations, about 90% of the carnitine filtered in the kidney is reabsorbed (Engel et al. 1981). The macronutrient content of human diets is known to affect both urinary excretion of carnitine and plasma carnitine concentrations. High fat diets have been shown to increase both plasma concentration and carnitine clearance over low fat diets (Cederblad 1987). Plasma carnitine concentrations tend to remain rather constant at a wide range of dietary intake, and it appears that renal adaptation to

carnitine intake is a major regulator of plasma carnitine concentration (Katrib et al. 1987). Persons given a diet high in fat, protein, or both excrete significantly increased amounts of carnitine (Rebouche et al. 1993). In that study, the increased carnitine excretion appeared to be the result of increased plasma carnitine concentration resulting an increased filtered load. Carnitine intakes were constant between groups so the diet apparently resulted in increase in either carnitine absorption, biosynthesis, or release from tissue stores.

Carnitine-Acyltransferases

Although carnitine was discovered as a nitrogenous compound of muscle tissue in 1905 (Fraenkel, G. and Friedman, S. 1905), it was not until 1955 that its function began to be understood. Fritz (1955) demonstrated that carnitine was the active component of muscle that was known to increase fatty acid oxidation in liver (Lundsgaard, E. 1950). In 1963 coenzyme A dependent formation of palmitoyl-carnitine in mitochondria was demonstrated (Bremer, J. 1962) as well as carnitine dependent oxidation of palmitoyl-coenzyme A with palmitoyl-carnitine as an intermediate (Fritz and Yue 1963).

As researchers isolated and characterized the carnitine acyl-transferases it became clear that there was more than one, based on substrate specificity (Bremer 1983). Although the issue is not yet resolved, at least three distinct enzymes are known to

exist, carnitine-acetyltransferase, carnitine-octanoyltransferase, and carnitine-palmitoyltransferase (Broquist 1994).

Carnitine-acetyltransferase

Carnitine-acetyltransferase (CAT) catalyzes the transfer of short-chain acyl groups from acyl-CoA to carnitine to make acylcarnitines. The K_m of CAT for acetyl-CoA is 37 μ M and nearly the same for other acyl-CoAs regardless of chain length. The reaction rate, however, in pigeon breast muscle (a common commercial source of CAT) is greatest for acetyl-CoA and decreases with increasing chain length of the acyl group with decanoyl-CoA having only about 4% of the activity of acetyl-CoA (Bremer 1983, Chase 1967, Chase and Tubbs 1966).

Depending on the species, CAT has a molecular weight ranging from 55,000 - 62 kDa (Bremer, 1983). Two forms of CAT are found in rat liver, mitochondrial and peroxisomal. The two are encoded by the same gene but have divergent sequences at the 5' end, the apparent result of alternative splicing mechanism which determines the subcellular localization of the gene product (Corti et al. 1994).

The functions of CAT are not clearly understood, but the most common function attributed to mitochondrial CAT is the regeneration of free CoA by the removal of the acyl groups from short-chain acyl CoA in the mitochondrial matrix (Broquist 1994). Another possible function is to facilitate the excretion of toxic short chain organic acids. Such short chain organic acids may be the result of normal energy metabolism,

abnormal energy metabolism (propionic acidemia and methylmalonic acidemia), or drug therapy (pivalic acid and valproic acid) and their excretion as carnitine esters, if excessive, can result in a secondary carnitine deficiency (Broquist 1994, Rebouche 1986).

Carnitine and acetylcarnitine have also been suspected of playing a role in the production of the neurotransmitter, acetylcholine, either directly or indirectly as a shuttle for acetyl groups from the mitochondria to the cytosol, where acetylcholine is formed (McCaman et al. 1966). Patients suffering with Alzheimer's disease have been shown to have decreased CAT activity in the brain (Kalaia and Harik 1992) and Alzheimer's patients treated with acetyl-carnitine have been shown to have a reduced progression of the disease (Spagnoli et al. 1991).

Whether the above roles for carnitine are essential or just facilitory has not been clearly established (Broquist 1994). The presence of CAT in high concentrations in muscles such as heart and flight muscles, which rely heavily on fat oxidation for energy, may suggest that it is essential for the mitochondrial regeneration of free CoA, however.

Carnitine-octanoyltransferase

Carnitine-octanoyltransferase (COT) is a 60 kDa protein that has been found in the mitochondria, peroxisomes and microsomes of mouse liver (Bremer 1983, Broquist 1994). It has a broad substrate range, overlapping with CAT and CPT. The

K_m of COT for octanoyl-CoA is $15\mu\text{M}$. Octanol-CoA is the best substrate for COT with acetyl-CoA and palmitoyl-CoA having reaction rates of 26% and 8 % of that of octanoyl-CoA (Bremer 1983). The functions of COT are less understood than for either CAT or CPT.

Carnitine-Palmitoyltransferases

There are two distinct CPT enzymes in cells, both of which reversibly catalyze the reaction $\text{Carnitine} + \text{Acyl-CoA} \rightarrow \text{Acyl-Carnitine} + \text{H-S-CoA}$ (Bremer 1983, Hoppel and Tomec 1971, Murthy and Pande 1994). One CPT, hereafter referred to as CPT-1, is a transmembrane protein located in the outer mitochondrial membrane. The other CPT, CPT-II, is bound to the inner surface of the inner mitochondrial membrane. Although both enzymes are capable of catalyzing the above reaction in both directions, under normal physiological conditions CPT-1 catalyzes the formation of acyl-carnitines from acyl-CoA's and CPT-II the reverse reaction (Figure 2-1).

CPT activity has also been located in peroxisomes and microsomes of cells (Bhuiyan et al. 1994, Murthy and Pande 1994) and appear to be distinct proteins from the mitochondrial CPT's. The function of peroxisomal and microsomal CPT has not been well characterized. It does not appear that either carnitine or CPT are required for the transport of acyl groups into peroxisomes; there is, however, evidence that carnitine may be involved in the shuttling of propionyl-CoA from peroxisomes to mitochondria for further oxidation (Jakobs and Wanders 1995). Because little is

known about non-mitochondrial CPT, only mitochondrial CPT will be discussed further.

Carnitine Palmitoyltransferase-I

CPT-1 occurs in at least two isoforms in rats, a liver CPT-1 (≈ 88 kDa) and skeletal muscle CPT-1 (≈ 82 kDa) (Britton et al. 1995). The cDNA for human liver CPT-1 has been recently cloned and sequenced and the genomic location determined to be on the q (long arm) of chromosome 11 (Britton et al. 1995). Mitochondrial CPT-I has been proposed to catalyze the rate limiting step in fatty acid oxidation, and regulation of its activity to be pivotal in the determination of energy substrate utilization (McGarry and Foster 1980). Brady et al. (1993) have suggested, however, that mitochondrial CPT-1 should not be considered rate limiting in fatty acid oxidation. They suggest that acyl-carnitine formation is actually rate limiting, and that acyl-carnitines may be the product of microsomal CPT as well as mitochondrial CPT. The application of molecular cloning techniques over the last 5 years has clarified two long-standing controversies regarding CPT. First, for many years it was debated if CPT-1 and CPT-2 were different proteins or if they were one protein with two different catalytic sites. It is now clear that they are two different proteins with separate functions and control mechanisms. Second, the issue of how CPT-I and CPT-II compare across tissue lines is partly resolved. It is now known that at least two different CPT-I isotypes exist. L-CPT-1, is found in liver, kidney, and

fibroblasts. M-CPT-I, is found in skeletal muscle, adipocytes, and is the predominant CPT-I in heart, although heart expresses both types. The two types of CPT-I are known to exhibit very different kinetics and inhibition sensitivities. The physiological implications of the different regulatory characteristics of CPT-I isotopes are just beginning to be understood (McGarry 1995).

CPT-1 is known to be regulated in 3 ways.

1. By malonyl CoA inhibition (Bremer 1983).
2. By changes in the sensitivity to malonyl CoA (Cook 1984, Cook et al. 1984).
3. By changes in the amount of the enzyme present, a result of changes in the rate of transcription and translation of the protein (Chatelain et al. 1996, Mynatt et al. 1994).

The inhibition of CPT-1 by malonyl CoA, the product of the first committed step in fatty acids synthesis, represents a negative regulation which insures that fatty acids are not being simultaneously synthesized and oxidized (Brady et al 1993). Changes in the malonyl CoA inhibition are a short-term regulatory mechanism, while increases in the rate of translation and transcription of CPT-1 represent a more sustained response, and is typically seen in starvation and diabetes (Brady et al 1993) . An unusual property of CPT-1 is that it is also inhibited by high concentrations of palmitoyl CoA, of one of its substrates (Bremer 1983, Zierz and Engel 1995).

CPT-1 has a reported apparent K_m for carnitine of $\approx 150 \mu\text{mol/L}$ (Murthy and Pande 1992, Kolodziej, M.P., et al. 1992), but shows considerable variation between

species and tissues with reported values as low as 32 $\mu\text{mol/L}$ for rat liver but 507 $\mu\text{mol/L}$ in muscle from the same animals, and 695 $\mu\text{mol/L}$ for dog heart (McGarry et al. 1983). The reported apparent K_m of CPT-1 for palmitoyl-CoA is considerably more uniform, at least in rat tissues, with 30 and 60 $\mu\text{mol/L}$ being reported for rat liver and skeletal muscle respectively (McGarry et al 1983).

Although CPT-1 in all tissues appears to be inhibited by malonyl CoA, there appears to be two distinct isotypes with different sensitivities to malonyl CoA. The CPT-1 of liver and kidney are one type whereas that of skeletal muscle and adipose tissue are a slightly smaller protein which is much more sensitive to malonyl CoA (Brady et al. 1993, Esserson et al. 1996, Saggerson et al. 1981,). Rat heart contains both liver and muscle CPT-1 (Brown et al. 1995). In neonatal rats the liver isoform contributes about 25% the total CPT-1 activity, but decreases with age to about 4% in adult animals.

Carnitine-Palmitoyltransferase-II

CPT-II occurs as only one isotype in all tissues and is $\approx 68\text{-kDa}$ in size (Brady et al 1993). As previously mentioned, CPT-II can catalyze bi-directionally. The CPT-II K_m for carnitine, however, is about 10 times higher than for CPT-I (1.5 mmol/L) assuring that the reaction will always predominate in the reverse direction under physiological conditions (Brady et al 1993). Unlike CPT-I, CPT-II is not inhibited by malonyl CoA, and all changes in activity appear to associated with corresponding

changes in the amount of protein present as well as the level of expression of its mRNA as determined by northern blots, and nuclear transcription rates as determined by nuclear run-on assays (Brady et al 1993). The human chromosomal CPT-II gene has not been yet been cloned (cDNA for the gene has been cloned) but it has been localized to the p32 region of chromosome 1 (Britton et al. 1995).

Interactions Between Nutrients

The concept of a nutrient interacting with another nutrient in ways that affect the tissue concentrations or dietary requirement for that nutrient is not new. It was reported in 1947 that dietary tryptophan reduces the dietary requirement for niacin (Krehl 1947, Young & Fukagawa 1988).

Interaction between vitamin C and iron:

A classical nutrient-nutrient interaction is that between vitamin C and iron. Vitamin C is able to increase the absorption of iron by keeping it in the reduced (Fe^{2+}) state, and by chelating it and keeping the iron in a more soluble form (Hallberg 1981). Vitamin C is also important in other aspects of iron metabolism such as synthesis of hemoglobin and the removal of iron from ferritin (Hallberg 1981).

Interaction between calcium and magnesium:

Similar nutrients often affect each other, such is the case with the two divalent cations calcium and magnesium. It has been reported that increasing the calcium consumption in humans can reduce absorption of dietary magnesium (Kim & Linkswiler 1980). In rats fed a parenteral nutrition diet with graded doses of calcium, urinary excretion of magnesium increased as calcium supplementation increased (Al-Jurf & Chapman-Furr 1985). Calcium, therefore, may decrease absorption and increase excretion of magnesium simultaneously.

Interaction between choline and carnitine:

Choline and carnitine are also very similar in structure as shown here.



With such striking similarity, it would not be surprising if interactions were found. Rats that are fed a choline deficient diet have been shown to have reduced carnitine concentrations in liver, heart, and skeletal muscle, but increased plasma carnitine concentrations compared to animals fed a choline supplemented diet (Carter & Frenkel 1978). Comparisons were not made to a control diet in this study.

Choline deficiency results in a decrease in lipid oxidation in heart and liver cells that can be reversed in vitro by the addition of carnitine (Corredor et al. 1967). The difference in carnitine levels in this study were even more dramatic after the animals were fasted for 48 h. Carnitine concentrations increased almost four fold in liver and significantly in heart and muscle after fasting of the choline supplemented animals. In the choline deficient animals carnitine increased in the liver about 3 fold but decreased only slightly in heart and muscle. It would appear that increases in carnitine concentrations are a part of the adaptation to fasting that is impaired when there is choline deficiency in rats.

It was shown in another study that the decreased hepatic carnitine in choline deficient rats results in a four fold decrease in oxidation of ^{14}C palmitate and an increased incorporation into triglyceride in liver homogenates (Corredor et al. 1967). Addition of carnitine, but not choline, in the liver homogenates restored palmitate oxidation to control levels and decreased the rate of esterification of palmitate to glycerol. This suggested that the fatty liver seen in choline deficient rats may be due in part to a lack of carnitine.

Two enzymes, choline-acetyltransferase and carnitine-acetyltransferase, also exhibit similarities, catalyzing the transfer of acetate from acetylcoenzyme A to choline and carnitine respectively. Carnitine-acetyltransferase has weak choline acetyltransferase activity, but choline-acetyltransferase is not cross reactive (Sastry & Janson 1994).

Choline and pantothenic acid supplementation has also been shown to reduce carnitine concentrations in plasma and 24 hr urine of humans (Mynatt et al. 1988, Dodson & Sachan, 1992). Follow-up studies demonstrated that the effect on carnitine was due to choline and not pantothenic acid (Sachan et al. 1993, Dodson and Sachan 1996). The results of these studies raised a number of intriguing questions which could not be adequately answered by reasonable experiments in humans. The questions included 1) Are the decreases in plasma and urine carnitine concentrations a reflection of choline-induced carnitine deficiency or an altered partitioning of carnitine among tissues? 2) Is choline effect mediated via the carnitine transporters in tissues? 3) What are the consequences of the decreased carnitine concentrations in plasma and urine?

A need for an animal model was clearly realized and a series of studies was initiated for the purpose of finding a good animal model to study the choline-carnitine interactions. An animal model was established, and is discussed in the next chapter.

Chapter 3

Preliminary Research and Objectives

The observation that choline and pantothenic acid supplementation resulted in a decrease in carnitine excretion to less than half that of unsupplemented subjects raised several interesting questions. First, was this a supplement induced carnitine deficiency, and if so which supplement? Some individuals had normal or higher plasma carnitine concentrations while others had lower concentrations, plasma data were inconsistent (Dodson and Sachan 1996). Supplemental carnitine, in both control subjects and those supplemented choline and pantothenic acid, increased urinary carnitine excretion above baseline values. However, carnitine excretion in the choline and pantothenic acid supplemented subjects was still about half that of controls.

There was no way of knowing how body carnitine pools other than urine and plasma were being affected, therefore an animal model was needed to study what effect choline was having on tissue concentrations and the total body pool of carnitine.

Rats were first used as an experimental model to study the interaction of choline and pantothenic acid on carnitine in humans. There was no effect of either choline, pantothenic acid, or both combined on either urinary excretion or plasma concentration of carnitine in rats (Daily and Sachan 1995). It was postulated that the

lack of effect in rats might be due to their high liver choline oxidase activity (Table 3-1). The animal with the choline oxidase activity nearest that of humans is the guinea pig. Guinea pigs were less convenient because of difficulty in drawing blood, but would hopefully respond similarly to humans when supplemented with choline.

Table 3-1
Liver Choline Oxidase in Selected Species

Species	Choline Oxidase Activity
Rat	2408 ± 121
Chicken	1311 ± 8
Mouse	895 ± 72
Hamster	361 ± 63
Monkey	144 ± 21
Guinea Pig	136 ± 43
Human	40 ± 7

¹Taken from Sidransky and Farber (1960). Activity units are in microliters of O₂ uptake/hr/g wet liver \pm SEM.

Pantothenic acid had no effect on either urinary excretion or plasma concentration of carnitine in guinea pigs (Daily and Sachan 1993). Choline supplementation (3g/kg diet), however, resulted in a significantly lowered carnitine excretion, similar to that seen in humans (Table 3-2). Plasma concentrations of carnitine were significantly higher in the choline supplemented group than in the non-supplemented group at the end of the study. This was different than what was seen in humans. Human studies had shown a trend toward a decrease in plasma carnitine concentrations with choline supplementation (Mynatt et al. 1988). A subsequent human study has clearly demonstrated, however, that choline affects human plasma carnitine concentrations differently for different individuals, some increasing and some decreasing (Dodson and Sachan 1996).

The decreased excretion of carnitine in humans and guinea pigs had several possible explanations. Since there was no consistent change in plasma concentrations of carnitine in humans, the decreased excretion would suggest a conservation of carnitine, or a decrease in available carnitine. One possibility was that absorption of dietary carnitine was impaired by choline supplementation. However, the carnitine content of the guinea pig diet was so low that it was not detectable, therefore, impaired absorption would have no significant affect on the guinea pigs if that were the cause.

Another possibility was that carnitine synthesis was impaired. However, it has never been shown that carnitine synthesis is impaired by choline. In fact,

Table 3-2
Effect of Choline Supplementation on Urinary Excretion
and Plasma Concentration of Carnitine in Guinea Pigs¹

Level of Choline Supplement	Carnitine			
	NEC	ASAC	AIAC	TOTAL ²
(g/kg diet)	Day 0 of Study, Urinary Excretion (μmol/d)			
0	1.3 ± 0.2	0.9 ± 0.2	0.1 ± 0.01	2.2 ± 0.3
3	1.5 ± 0.2	0.8 ± 0.2	0.1 ± 0.03	2.4 ± 0.3
	Day 5 of Study, Urinary Excretion (μmol/d)			
0	1.5 ± 0.2	0.6 ± 0.1	0.1 ± 0.01	2.6 ± 0.4
3	0.4 ± 0.1*	0.3 ± 0.0*	0.1 ± 0.01	0.8 ± 0.1*
	Day 10 of Study, Urinary Excretion (μmol/d)			
0	1.7 ± 0.1	0.8 ± 0.2	0.1 ± 0.01	2.5 ± 0.3
3	0.5 ± 0.1*	0.5 ± 0.1	0.1 ± 0.01	1.1 ± 0.1*
	Day 10 of Study, Plasma Concentration (μmol/L)			
0	38.1 ± 2.1	16.1 ± 4.3	1.1 ± 0.2	54.4 ± 4.5
3	47.2 ± 1.8*	30.8 ± 6.0	0.9 ± 0.1	74.0 ± 5.9*

¹Values are means ± SEM for the groups. Guinea pigs were fed ground diet with either no supplement or 3 g/kg choline from choline chloride for 10 days (diet contained 1.85g/kg choline without added supplement). On d 0 (pre-experimental diet) and d 5, n = 12 for both groups; on day 10, n = 6 for the choline supplemented group and n = 6 for the non-supplemented group.

An asterisk indicates significant difference between the supplemented and non-supplemented groups (P ≤ 0.05) by Student's t test.

²NEC = non-esterified carnitine, ASAC = acid-soluble acyl carnitine, AIAC = acid-insoluble acyl carnitine, Total = total carnitine (Daily and Sachan 1995).

just the opposite occurs in rats, choline deficiency impairs carnitine synthesis (Carter & Frenkel 1978). Comparing carnitine biosynthesis between groups in a definitive manner is not possible. Frequently, rates of synthesis can be compared by comparing the activity of biosynthetic enzymes. Carnitine biosynthesis, however, does not appear to be enzymatically regulated. Rather, it appears to be regulated by release of trimethyllysine as proteins are broken down (Sandor & Hoppel 1989, Sandor 1990). If decreased carnitine biosynthesis is responsible for the decreased excretion of carnitine in choline supplemented humans and guinea pigs, that might be reflected in decreased tissue concentrations of carnitine.

To explore that possibility, another group of guinea pigs was studied for the purpose of obtaining more tissue samples. Blood collections were taken during the course of this study, instead of just at the end. Urinary excretion of free and total carnitines were reduced in the choline supplemented group as before, but acyl carnitines were elevated in both groups but more so in the choline supplemented group (Daily and Sachan 1995). No tissue carnitine concentrations were lower in the choline supplemented group, and the carnitine concentration was significantly higher in skeletal muscle (**Figure 3-1**). This demonstrated conclusively that there was not a choline-induced carnitine deficiency in guinea pigs.

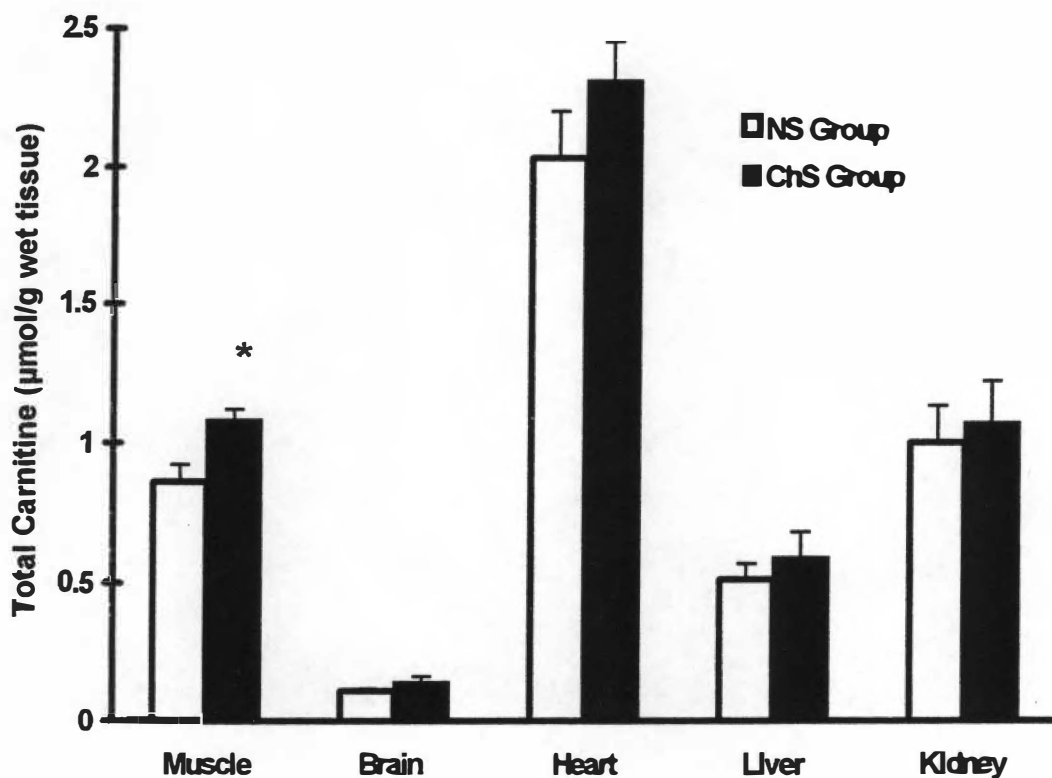


Figure 3-1: Effect of choline supplementation on tissue concentrations of total carnitine in guinea pigs. Each group was fed guinea pig diet either supplemented (ChS group) with choline (3g/kg diet) or with no supplement (NS group). Values are group means \pm SEM (n = 12). Significant difference between groups by Student's t test is indicated by an asterisk (Daily & Sachan, 1995).

Plasma β -hydroxybutyrate was also higher in the choline supplemented guinea pigs (Daily & Sachan 1995). This raised two questions. First, were the serial blood collections (by cardiocentesis) under anesthesia responsible for the increase in acyl carnitine and β -hydroxybutyrate? This was later confirmed in a study of the effect of anesthesia and cardiocentesis on urinary excretion (Park et al. 1995, Park et al. 1996). Second, if the anesthesia and blood collection were responsible, did the higher plasma β -hydroxybutyrate and urinary acyl-carnitine indicate that choline supplemented guinea pigs have an increased capacity for fatty acid oxidation?

Only one dose of carnitine in humans (20mg/kg body weight) and another in guinea pigs (3g/kg diet) had been used in these studies. The human dose was based on the amount used by the individual in which the choline effect was first observed (Dodson and Sachan 1996, Mynatt et al. 1988). The dose used in guinea pigs represented a similar percent increase over normal dietary choline as was shown to be effective in humans, thereby demonstrating a similar effect with similar increases in choline intake. What level of choline supplementation is required to be effective, and how much is required for a maximal effect, remained unanswered.

The following studies were designed to address: 1) the possible functional consequences of this newly discovered choline-carnitine interaction on fatty acid oxidation in guinea pigs, and 2) the minimal choline dose required to obtain the effect on carnitine status, and the dose which yields the maximal effect.

Chapter 4

Effect of Varied Choline Doses on Plasma, Urinary and Tissue Carnitine in Guinea Pigs

Introduction

Choline and carnitine are two trimethylated quaternary amines that are very similar in structure, but without many similarities in function. Both use different transport proteins for intestinal and renal absorption. Both can be acetylated by similar CoA-dependent acetyl-transferase enzymes, but for different purposes. In the past, studies of choline-carnitine interactions have evaluated the effect of choline deficiency on carnitine status (Aarsaether et al 1988, Carter and Frenkel 1978, Corredor et al 1967). These studies have demonstrated that choline deficiency in rats result in a decrease in liver, heart and skeletal muscle carnitine, and that fatty acid oxidation is impaired. We, however, have demonstrated an effect of choline supplementation in excess of normal dietary intake on carnitine homeostasis (Daily and Sachan 1995, Dodson and Sachan 1996). Choline supplementation in humans (20 mg/kg body weight) and guinea pigs (3 g/kg diet) results in a decrease in urinary excretion of carnitine to less than half normal values. In these studies, plasma carnitine concentrations were variably affected in humans, but were increased in guinea pigs.

Tissue concentrations of carnitine were also affected in guinea pigs, with choline supplementation causing a significant increase in skeletal muscle carnitine; however, tissue carnitine concentrations have not yet been determined in humans.

The choline dose in the above human study was based on what was observed in a person using a choline supplement in which the effect on carnitine was first observed (Dodson and Sachan, 1996). The levels of choline supplementation in guinea pigs reflected a similar increase over normal dietary choline levels (the level of supplement was approximately 1.7 times that found in the diet). In neither species, however, has either a minimal effective choline dose or the level of choline supplementation for maximum effect been determined. The primary objective of this study was to determine such dose effects in guinea pigs.

Low carnitine excretion in humans is frequently the result of a low dietary intake of carnitine. Low dietary intake of carnitine in choline supplemented guinea pigs can not be the reason for reduced excretion of carnitine because the guinea pig diet is essentially carnitine free (Daily and Sachan 1995). It is possible that if carnitine were being lost from the intestinal tissue, excreted into the intestinal lumen, and then not reabsorbed, that a reduced urinary carnitine excretion could be a compensatory mechanism to preserve carnitine. An intestinal loss into the lumen would be expected to result in a decrease in gut tissue carnitine with a simultaneous increase in carnitine in the lumen contents, and possibly fecal carnitine. Therefore, a secondary objective was to evaluate the likelihood of intestinal loss of carnitine by determining its

concentrations in intestinal tissue, lumen contents, and feces. Additional secondary objectives of these studies were to 1) determine the effect of choline on total body carnitine, 2) determine the effect of choline on body composition, and 3) determine the effect of choline on feed efficiency in guinea pigs.

Materials and Methods

All research protocol for these studies were approved by the Animal Care and Use Committee of the University of Tennessee. Prior to the dose response study an attempt was made to use a semi-purified diet for the dose response study. Ten male, nine week old, Hartley guinea pigs (SASCO, Inc; Omaha, NE) were divided into two groups of five and fed either ground commercial guinea pig diet (Purina Guinea Pig Chow no. 5025, Purina Mills, Richmond, IN) or a semi-purified diet (**Table 4-1**). With one exception, the guinea pigs did not eat the semi-purified diet, and the one that did eat, consumed less than the animals on the control diet (**Table 4-2**). The self-induced starvation resulted in an average weight loss of almost 40 grams in 5 days, during the same period the animals on the commercial diet gained an average of about 30 grams. For one day, the semi-purified diet was supplemented with 4% alfalfa flour, but this resulted in only a small change in acceptance of the semi-purified diet by one animal (**Table 4-2**). It was decided that the guinea pigs would not eat a semi-purified diet normally, and that using a choline supplemented commercial diet, as used in previous

Table 4-1
Composition of Guinea Pig Semi-Purified Diet

Ingredient	g/kg Diet
Soy Protein	200.00
L-Arginine	3.00
D,L-Methionine	5.00
Corn Oil	50.00
Corn Starch	150.00
Sucrose	140.00
Glucose	120.00
Mineral Mix	67.50
AIN Vitamin Mix	10.00
Ascorbate	1.00
Choline Chloride	2.47
Alphacel	251.03

Table 4-2
Food Consumption and Weight Gain or Loss in Guinea Pigs
Fed Ground Commercial Diet or Semi-Purified Diet¹

	Groups	
	Commercial Diet	Semi-Purified Diet
Feed Consumption (g/d) Prior to Experimental Diets	30.80 \pm 1.10	30.90 \pm 0.84
Feed Consumption (g/d) While on Experimental Diets	31.24 \pm 1.61	5.56 \pm 1.80
Feed Consumption (g/d) While on Semi-Purified Diets With 4% Alfalfa Added		8.00 \pm 4.59
Weight Change (grams) During Experimental Period	+ 29.6 \pm 7.22	- 39.4 \pm 12.77

¹Values shown are the means for the group over 5 days \pm SEM.

studies, would be more satisfactory. Because this was a dose response study, it would be undesirable for daily choline intake to be determined by the willingness of animals to eat normally rather than the choline concentration of the diet. Other researchers have also experienced difficulty in getting guinea pigs to eat semi-purified diets. For example, Teo et. al. (1991) reported that weanling male Hartley guinea pigs had to be selected for their adaptability to a semi-purified diet.

Of the ten guinea pigs, 2 were transferred to another study and the 8 remaining were divided into four groups of two and used for the first of two trials in the dose response study. After completing the study with the first 8 guinea pigs, 12 additional male Hartley guinea pigs (5 week old) were purchased and divided into 4 groups of 3 to complete the study with a total of 20 guinea pigs. All animals were individually housed in stainless steel wire mesh cages except when urine was being collected. All animals were allowed free access to water and feed (Purina Guinea Pig Chow no. 5025, Purina Mills, Richmond, IN) for one week prior to the beginning of the study, and to water and their respective diets during the study .

There were four groups of five guinea pigs assigned to one of the four doses of dietary choline supplement. Experimental diets were prepared by grinding guinea pig diet and adding either no choline or 2, 5, or 10 g choline from choline chloride per kg diet (**Table 4-3**). Since the diet contains endogenous choline of approximately 1.85 g/kg diet, this represents a range from approximately 2 - 12 g/kg diet.

Table 4-3
Choline Content of Control and Experimental Guinea Pig Diets

	Experimental Groups			
	NS	2ChS	5ChS	10ChS
Choline from Diet (g/kg)	1.85	1.85	1.85	1.85
Choline Added to Diet (g/kg)	0.00	2.00	5.00	10.00
Total Choline in Diet (g/kg)	1.85	3.85	6.85	11.85

The first set of guinea pigs included those that had been on the semi-purified diets and began the study after the group on semi-purified diets regained their weight and were comparable in weight with the guinea pigs fed the commercial diet. The second set of guinea pigs was placed on experimental diets one week after arrival.

Throughout the study, records were kept of food consumption and weight gain. Feed efficiency was calculated as the amount of weight gained in grams per gram of feed eaten (g weight gain/g feed eaten). Urine was collected before beginning the experimental diets and on days 2, 5, 14, and 21 and feces collected on day 21 of the dietary treatment. Urine was collected by placing the animals in acrylic metabolic cages for 24 hours with a urine collector containing 1 ml of 0.2 mol/L HCl. Blood was collected by cardiac puncture under open drop methoxyflurane anesthesia on day 21. The blood was centrifuged and the plasma frozen at -20°C until assayed. Following the blood collection, the guinea pigs were killed by decapitation while still under

anesthesia. The small intestine was clamped at the ileal and duodenal ends, surgically removed and the lumen contents flushed twice with 10 ml of saline and the flush saved for carnitine assay. The intestine was then divided into 3 segments and each segment measured, weighed, and frozen at -80°C until assayed for carnitine. Heart, brain, kidneys, liver, and gastrocnemius muscle were also removed, and stored at -80°C for carnitine assay. Animal carcasses were saved and frozen at -80°C for proximate analysis and total body carnitine determination.

Carnitine Assay:

Carnitine was assayed in blood, urine, and tissue samples by a modified radioisotopic method of Cederblad and Linstedt (Cederblad and Lindstedt 1972, Sachan et al. 1984). In this assay, acid insoluble long-chain acyl carnitines (AIAC) are precipitated using perchloric acid, leaving the short-chain acid soluble acyl carnitines (ASAC) and the non-esterified carnitines (NEC) in the supernatant. An aliquot of the supernatant is assayed to determine the NEC concentration and another hydrolyzed with 0.5 N KOH to assay all acid soluble carnitines. ASAC is the difference between the total acid soluble carnitines and the NEC. The pellets are drained, washed, suspended in 0.5 N KOH, and hydrolyzed in a water bath at 60°C for 60 minutes and assayed for carnitine.

In each case carnitine is determined by using carnitine acetyl transferase (Sigma Chemical Co., St Louis, MO) to esterify carnitine to a ^{14}C -acetate from 1, ^{14}C -acetyl

CoA (Moravek Biochemicals, Brea, CA). Unreacted ^{14}C -acetyl CoA is removed from the reaction mixture by running the reaction mixture through anion exchange columns and the radioactivity of samples and standards determined using a Beckman LS3801 liquid scintillation counter.

Total body carnitine was estimated by determining carnitine in liquid carcass homogenates, and adding to that amount the amount of carnitine removed with organs and tissues. Organ and tissue carnitine was determined by multiplying the carnitine concentration by the weight of the tissue. This method probably resulted in a small underestimate of total body carnitine since the large intestine, stomach, and caecum were removed but not assayed for carnitine.

Creatinine and Renal Function:

Creatinine assays in urine were performed by the Jaffe reaction according to the methods of Taussky (1954) as modified by Henry (1967). Plasma creatinine was determined using another modification of the Jaffe reaction by Raffael (1976). Creatinine clearance was calculated from plasma concentration and 24h urinary excretion of creatinine, using the equation: $\text{Clearance} = (U_x/P_x)V$ where U_x = 24 h urinary creatinine excretion, P_x = plasma creatinine concentration, and V = urine flow in ml per minute. Carnitine clearance was calculated by the same equation except U_x and P_x = are 24 h urinary carnitine excretion and plasma carnitine concentration, respectively.

Fractional tubular reabsorption (FTR) of carnitine was calculated by the formula (Zamora et al. 1995):

$$FTR\% = \left\{ 1 - \frac{Ucne}{Pcne} \times \frac{Pcreat}{Ucreat} \right\} \times 100$$

Where FTR = fractional tubular reabsorption, Ucne = 24 h urinary excretion of carnitine ($\mu\text{mol/d}$), Pcne = plasma concentration of carnitine ($\mu\text{mol/L}$), Pcreat = plasma concentration of creatinine ($\mu\text{mol/L}$), and Ucreat = 24 h urinary excretion of creatinine ($\mu\text{mol/d}$).

Proximate analysis of Guinea Pig Carcasses

Guinea pig carcasses were thawed and shaven to remove hair. Large intestines, stomach, and caecum were removed. The carcasses were homogenized in a blender and the homogenate lyophilized for moisture determination. Aliquots of the dried homogenate were assayed for protein by the kjeldahl method (Official Methods of Analysis 1984). Percent fat was determined by soxhlet extraction for 12 h using hexane as the solvent. The percent fat in the sample was determined both by the amount of the fat recovered in the flask, and by the loss of weight from the sample, after extraction. The values obtained from the two methods were averaged for the

reported fat percent. Mineral ash percent was determined by ashing an aliquot of the lyophilized sample in a muffle furnace at 650⁰ C.

Statistics

All values are expressed as group means \pm SEM. Urinary carnitine excretion was expressed as μ mol carnitine excreted per mmol creatinine. Significance of differences were determined using analysis of variance (ANOVA) and Tukey's test. Statmost for Windows version 2.01 (DataMost Corp., Salt Lake City, UT) was used for ANOVA. For urinary excretion of carnitine differences were evaluated using a repeated measures ANOVA using the MIXED procedure of SAS (Cary, N.C.). When the F test indicated differences between groups, the differences were separated using Fisher's protected least significant difference test. For regression analysis the regression curve was generated using Excel for Windows version 5.0 and regression analysis performed using SAS (Cary, NC).

Results

The difference in the ages of the two sets of guinea pigs introduced enough variation in body weights, food consumption, and weight gain so that these data from the two sets could not be meaningfully combined for analysis. There were no significant differences among the dietary groups in initial and body weight, weight gain, feed consumption, or feed efficiency in either set of animals (**Table 4-4**).

Urinary excretion of carnitine was significantly lower in all three groups of choline supplemented guinea pigs than in the nonsupplemented group on day 5 and for the remainder of the study (**Table 4-5**). There was a time dependent increase in urinary carnitine excretion in the nonsupplemented group, that was not seen in the supplemented groups. There was no apparent dose effect during that period since the different levels of choline supplementation did not result in significantly different urinary excretions of carnitine. On day 2, the excretion of the carnitine in the animals supplemented with 5 and 10 g choline per kg diet was approximately half that of the unsupplemented animals, although this difference was not statistically significant.

Carnitine concentration was determined in plasma, kidney, brain, liver, heart, and skeletal muscle (**Table 4-6**). Plasma, brain, and liver concentrations of carnitine were not significantly different between any of the groups. Kidney free carnitine was higher

Table 4-4
Feed Consumption, Weight Gain, and Feed Efficiency in
Choline Supplemented and Non-Supplemented Guinea Pigs¹

Level of Choline Supplement (g/kg diet)	0	2	5	10
First Set of Guinea Pigs				
Beginning Weight	926.0	888.0	914.5	895.5
Final Weight	930.5	914.0	914.5	914.5
Weight Gain	4.5	26.0	0.0	19.0
Feed Eaten	938.0	838.5	939.0	856.0
Feed Efficiency	0.0068	0.0339	0.0002	0.0248
Second Set of Guinea Pigs				
Beginning Weight	300.7 ± 20.5	302.7 ± 3.8	314.3 ± 11.6	298.3 ± 17.0
Final Weight	402.7 ± 35.4	421.7 ± 9.7	431.7 ± 9.6	405.3 ± 15.3
Weight Gain	102.0 ± 17.0	119.0 ± 9.0	117.3 ± 14.4	107.0 ± 4.2
Feed Eaten	513.0 ± 31.5	556.3 ± 25.3	573.7 ± 39.3	500.0 ± 24.9
Feed Efficiency	0.1962 ± 0.021	0.2134 ± 0.007	0.2031 ± 0.011	0.2148 ± 0.011

¹Values are group means (± SEM for the second set of guinea pigs) (n = 2 for the first set and n = 3 for the second set). Differences between groups were evaluated by ANOVA test but differences were not significant (P < 0.05).

Table 4-5
Urinary Excretion of Carnitine in Guinea Pigs Supplemented
With Varied Levels of Choline for 21 Days¹

Level of Choline Supplement	Carnitine ($\mu\text{mol}/\text{mmol}$ Creatinine)			
	NEC	ASAC	AIAC	TOTAL ²
(g/kg diet)	Day 0 of Study			
0	14.0*	8.5	0.7*	23.2*
2	12.6	9.8	0.4*	22.8
5	19.9	11.0	0.4*	31.4
10	21.9*	11.8	0.6*††	34.3*
	Day 2 of Study			
0	32.2 ^{a,†}	12.5	0.9*	45.5 [†]
2	27.4 ^{ab}	14.7	0.8*	42.7
5	11.4 ^b	10.4	0.3*	22.0
10	14.6 ^{b,*†}	15.5	0.3*†	30.4*†
	Day 5 of Study			
0	31.4 ^{a,†}	16.5	0.4*	48.3 ^{a,†}
2	14.1 ^{ab}	9.8	0.3*	24.2 ^b
5	7.6 ^{bc}	7.6	0.2*	15.4 ^b
10	5.9 ^{c,†}	5.8	0.2*	12.3 ^{b,†}

Continued

Table 4-5 Continued

Level of Choline Supplement	Carnitine ($\mu\text{mol}/\text{mmol}$ Creatinine)			
	NEC	ASAC	AIAC	TOTAL
(g/kg diet)	Day 14 of Study			
0	52.7 ^{a,†}	18.6	3.2 ^{a,†}	74.8 ^{a,†}
2	12.4 ^b	10.2	1.5 ^{b,†}	24.1 ^b
5	20.4 ^b	14.7	1.5 ^{b,†}	37.7 ^b
10	16.3 ^{b,*†}	16.5	1.2 [‡]	36.5 ^{b,*}
	Day 21 of Study			
0	40.5 ^{a,†‡}	17.8	2.8 ^{a,†}	61.1 ^{a,†‡}
2	18.7 ^b	11.8	1.3 ^{b,†}	31.7 ^b
5	18.4 ^b	11.4	1.6 ^{b,†}	31.4 ^b
10	9.9 ^{b,*†}	7.8	1.0 ^{b,†‡}	18.6 ^{b,*†}
Pooled Standard Error	6.5	3.3	0.3	8.5

¹Values are means for the group, pooled standard error for carnitine fractions are shown at the bottom of the table. Values in a column, for each day of the study, with a different letter superscript (a,b,c) are significantly different from others in the same column at the same time period ($P < 0.05$, $n = 5$). Differences over time within supplement groups, for each column, are indicated by different symbol superscripts (*,†,‡), ($P < 0.05$, $n = 5$).

²NEC = non-esterified carnitine, ASAC = acid-soluble acyl carnitine, AIAC = acid-insoluble acyl carnitine, Total = total carnitine.

Table 4-6
Plasma and Tissue Concentrations of Carnitine in Guinea Pigs
Supplemented With Varied Levels of Choline for 21 Days¹

Level of Choline Supplement	Carnitine			
	NEC	ASAC	AIAC	TOTAL
(g/kg diet)	Plasma (μmol/L)			
0	33.1 ± 6.0	2.6 ± 1.2	2.2 ± 0.7	37.9 ± 6.6
2	40.4 ± 4.0	9.1 ± 3.4	2.9 ± 0.6	52.3 ± 4.5
5	36.6 ± 8.3	8.9 ± 2.5	1.3 ± 0.5	46.8 ± 11.2
10	15.6 ± 2.1	25.2 ± 7.3	2.0 ± 0.6	42.8 ± 6.3
	Brain (nmol/g)			
0	131.7 ± 24.2	31.0 ± 145.0	43.1 ± 7.6	205.8 ± 44.3
2	211.3 ± 54.5	39.3 ± 16.4	41.2 ± 4.8	291.8 ± 70.0
5	154.1 ± 9.7	26.5 ± 6.6	34.5 ± 2.7	215.1 ± 12.1
10	208.1 ± 51.4	26.0 ± 9.9	46.2 ± 9.9	280.3 ± 63.7
	Liver (nmol/g)			
0	326.3 ± 48.9	5.3 ± 5.3	12.5 ± 1.1	344.1 ± 50.5
2	341.0 ± 34.4	12.8 ± 5.3	11.3 ± 2.5	365.1 ± 38.9
5	359.0 ± 32.9	21.8 ± 10.3	8.6 ± 2.0	389.4 ± 42.8
10	357.0 ± 43.4	17.9 ± 10.6	13.2 ± 3.8	388.2 ± 62.6

Continued

Table 4-6 - Continued

Level of Choline Supplement	Carnitine			
	NEC	ASAC	AIAC	TOTAL
<hr/>				
(g/kg diet)	Kidney (nmol/g)			
0	379.8 ± 19.5 ^a	55.6 ± 13.9	15.4 ± 2.5 ^a	450.8 ± 18.6
2	397.1 ± 18.4 ^{ab}	59.6 ± 7.0	17.3 ± 0.9 ^{ab}	474.0 ± 19.0
5	391.5 ± 24.3 ^{ab}	68.6 ± 22.4	21.0 ± 1.5 ^b	481.1 ± 21.2
10	428.1 ± 19.3 ^b	39.0 ± 6.5	18.7 ± 1.1 ^{ab}	485.7 ± 21.6
<hr/>				
	Heart (nmol/g)			
0	1042.5 ± 145.9 ^a	479.6 ± 33.1	23.2 ± 12.7 ^a	1545.2 ± 140.7 ^a
2	1311.2 ± 108.2 ^{ab}	505.5 ± 67.1	50.8 ± 8.3 ^{ab}	1867.4 ± 130.5 ^b
5	1373.0 ± 141.8 ^{bc}	479.6 ± 61.2	88.4 ± 7.9 ^b	1941.0 ± 153.1 ^b
10	1063.0 ± 90.5 ^a	395.4 ± 72.0	87.2 ± 29.8 ^b	1545.6 ± 28.2 ^a
<hr/>				
	Skeletal Muscle (nmol/g)			
0	544.9 ± 25.0	48.7 ± 14.7 ^a	217.3 ± 27.9	810.9 ± 27.6 ^a
2	635.0 ± 39.7	96.2 ± 33.4 ^a	251.8 ± 42.4	983.0 ± 51.6 ^b
5	608.9 ± 56.9	103.0 ± 16.1 ^a	230.1 ± 18.6	942.2 ± 65.5 ^{ab}
10	624.1 ± 118.6	191.2 ± 29.9 ^b	231.0 ± 64.7	1046.3 ± 117.3 ^b

¹Tissue concentrations are nmol/g wet tissue. Values are expressed as means for the group (n=5) ± SEM. Values in a column with a different superscript are significantly different (P < 0.05) by ANOVA and Tukey's test.

²NEC = non-esterified carnitine, ASAC = acid-soluble acyl carnitine, AIAC = acid-insoluble acyl carnitine, Total = total carnitine.

in the group supplemented with 10 g choline per kg diet, but there was no significant trend in the other fractions or in total carnitine. In the heart muscle, the AIAC was elevated in all choline supplemented animals. In the groups supplemented with 2 and 10 g choline per kg diet, both free and total carnitine were significantly higher than in the nonsupplemented animals. There were no differences in heart ASAC between groups. Heart carnitine concentrations were not different for any fractions except AIAC or for total carnitine in the group supplemented with 10 g choline per kg diet. All choline supplemented groups of guinea pigs had higher total carnitine concentrations in skeletal muscle. Although there appeared to be a dose dependent increase in ASAC in muscle of choline supplemented animals, differences were significant only in the 10 g choline per kg diet group. Carnitine concentrations in small intestinal tissue are shown in **Figure 4-1**. In all segments and in the whole intestine, there was an apparent dose dependent increase in total carnitine concentrations in choline supplemented animals, but was significant only in the group getting 10 g choline per kg diet. Differences in free or acyl carnitines were not significant between groups. Although the differences were not significant, there was an apparent increase in both free and acyl carnitines that, when combined, resulted in a significantly higher total carnitine. The carnitine concentration of the lumen contents of the small intestine and feces were highly variable, and there were no differences among groups (**Table 4-7**). Body composition, especially % fat, was affected more by age than by the choline supplement (**Table 4-8**). Older animals had a much higher % body fat than did the

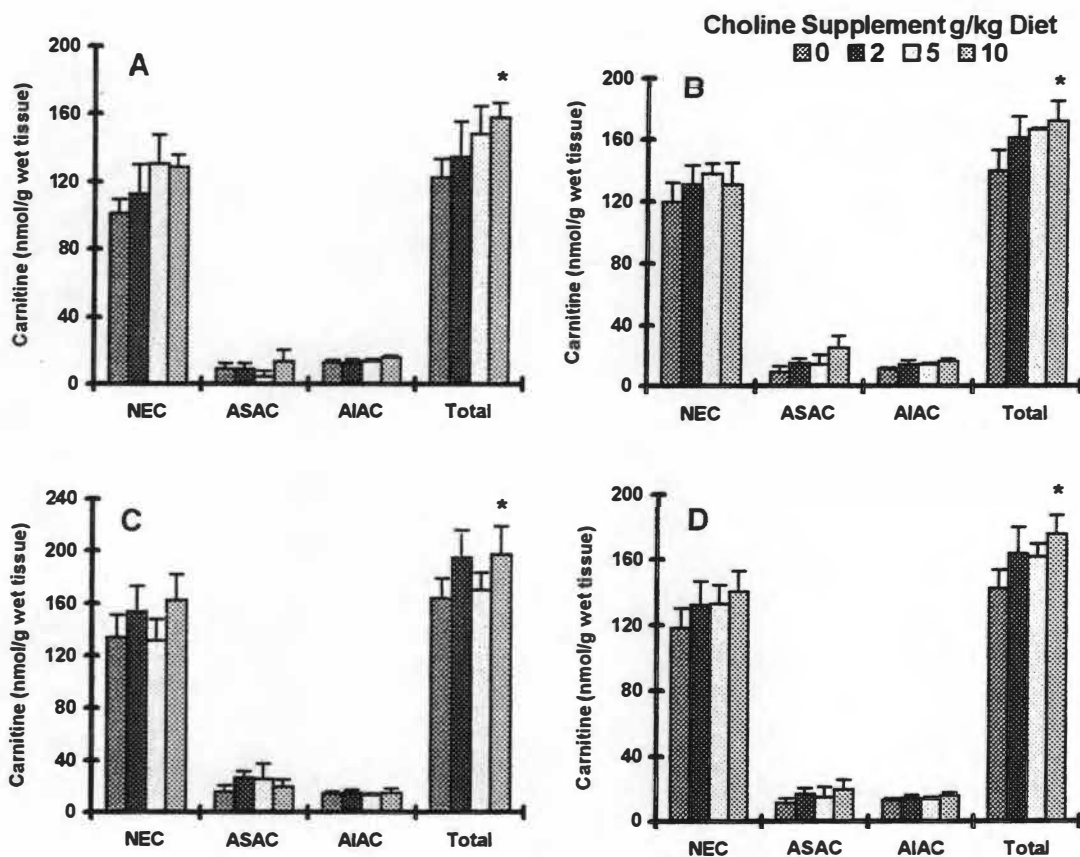


Figure 4-1: Carnitine concentrations in proximal (A), middle (B), distal (C), or average of all segments (D) of the small intestine of guinea pigs given varied levels or no supplemental choline added to ground diet for 21 days. NEC = non-esterified carnitine, ASAC = acid-soluble acyl carnitine, AIAC = acid insoluble acyl carnitine, and Total = total carnitine. All values are the group mean ($n = 5$) \pm SEM. Significant difference ($P < 0.05$) from the control group (0 g/kg diet) by ANOVA Tukey's test is indicated by an asterisk.

Table 4-7
Intestinal Lumen Content and Feces Concentrations of Carnitine in Guinea
Pigs Supplemented With Varied Levels of Choline for 21 Days¹

Level of Choline Supplement	Carnitine			
	NEC	ASAC	AIAC	TOTAL
(g/kg diet)	Small Intestine Lumen Content (nmol/animal)			
0	93.96 ± 60.86	49.59 ± 0.55	20.66 ± 10.09	164.22 ± 87.98
2	41.50 ± 7.66	39.21 ± 12.23	15.32 ± 3.60	96.03 ± 20.73
5	58.61 ± 21.8	55.77 ± 1.37	23.25 ± 11.29	134.42 ± 44.39
10	65.73 ± 16.80	60.66 ± 10.02	13.30 ± 3.69	139.69 ± 5.24
	Feces (nmol/g)			
0	5.47 ± 15.4	0.80 ± 0.45	1.51 ± 0.95	7.61 ± 2.64
2	6.14 ± 1.36	1.02 ± 0.55	1.05 ± 0.03	8.21 ± 1.59
5	4.31 ± 1.15	1.51 ± 0.79	0.45 ± 0.19	6.80 ± 1.88
10	3.30 ± 0.69	0.53 ± 0.17	0.53 ± 0.19	4.37 ± 0.66

¹Values are expressed as means for the group (n=5) ± SEM. None of the means between groups are significantly different (P < 0.05) by ANOVA.

²NEC = non-esterified carnitine, ASAC = acid-soluble acyl carnitine, AIAC = acid-insoluble acyl carnitine, Total = total carnitine.

Table 4-8
Body Composition of Guinea Pigs Supplemented with
Varied Levels of Choline for 21 Days¹

	Level of Choline Supplement (g/kg diet)			
	0	2	5	10
Both Sets of Guinea Pigs Combined (n = 5)				
Moisture %	60.8 ± 2.8	62.4 ± 3.4	60.5 ± 3.2	63.4 ± 3.7
Fat %	16.2 ± 3.5	15.3 ± 3.6	15.6 ± 3.1	14.2 ± 3.9
Protein %	15.4 ± 0.9	16.1 ± 0.7	16.5 ± 0.9	16.1 ± 0.7
Ash %	4.3 ± 0.3	4.0 ± 0.2	4.6 ± 0.2	4.7 ± 0.3
Total %	96.8 ± 0.8	97.8 ± 1.0	97.1 ± 1.0	98.4 ± 0.9
Ratio %Fat / %Protein	1.05 ± 0.23	0.97 ± 0.25	1.00 ± 0.25	0.91 ± 0.27
First Set of Guinea Pigs (n = 2)				
Fat %	24.3	24.0	23.2	23.5
Protein %	15.3	16.0	14.6	15.1
Ratio %Fat / %Protein	1.59	1.53	1.63	1.56
Second Set of Guinea Pigs (n = 3)				
Fat %	10.8 ± 1.1	9.6 ± 1.1	10.6 ± 1.1	8.1 ± 1.1
Protein %	15.4 ± 1.0	16.2 ± 1.0	17.7 ± 1.0	16.8 ± 1.1
Ratio %Fat / %Protein	0.69 ± 0.02 ^a	0.59 ± 0.08 ^{ab}	0.60 ± 0.05 ^{ab}	0.48 ± 0.07 ^b

¹Proximate analysis was performed on whole body homogenates of guinea pig fed 4 levels of supplemental choline in ground commercial diet. Total % is the combined percentages of moisture, fat, protein, and ash for each group. Each value represents the mean for the group (± SEM, n = 5 for the combined data, and second set of guinea pigs). Different superscripts indicate significant differences by ANOVA and Tukey's test (P < 0.05).

younger animals. The % protein in the carcasses was similar between the two sets of animals, but the younger animals had a higher % of moisture than the older animals (66% vs 54%). Comparing the body composition data by ANOVA and Tukey's test revealed no significant differences between groups in any of the measurements of body composition, when the two sets of animals were considered as a whole. When the two sets were analyzed separately. The ratio of %fat / %protein was significantly lower in the animals supplemented with 10 g choline per kg diet than in the nonsupplemented animals in the second (younger) set of animals. However, ANOVA is problematic in comparing these groups. When the two sets are compared separately, there is an n of 2 in one set and 3 in the other; normally too small to be adequate for ANOVA. When the two sets are combined, the variation in fat % between the two sets is so large that too much variation is introduced by the age effect to permit differences to be significant for choline supplementation. Regression analysis (**Figure 4-2**) was also used for the data from the second set of guinea pigs to determine if choline dose could be responsible for an apparent effect on %fat and the ratio of %fat / %protein, which was used as an indicator of fat/lean body mass. There was not a significant decrease in %fat with increasing choline supplementation ($R^2 = 0.23$, $P = 0.1$). There was a significant trend ($R^2 = 0.47$, $P = 0.01$) for a decrease in the ratio of %fat/%protein with increasing choline in the second set of animals, suggesting that the variation in choline dosage was responsible for 47% of the variation in the ratio of fat/protein.

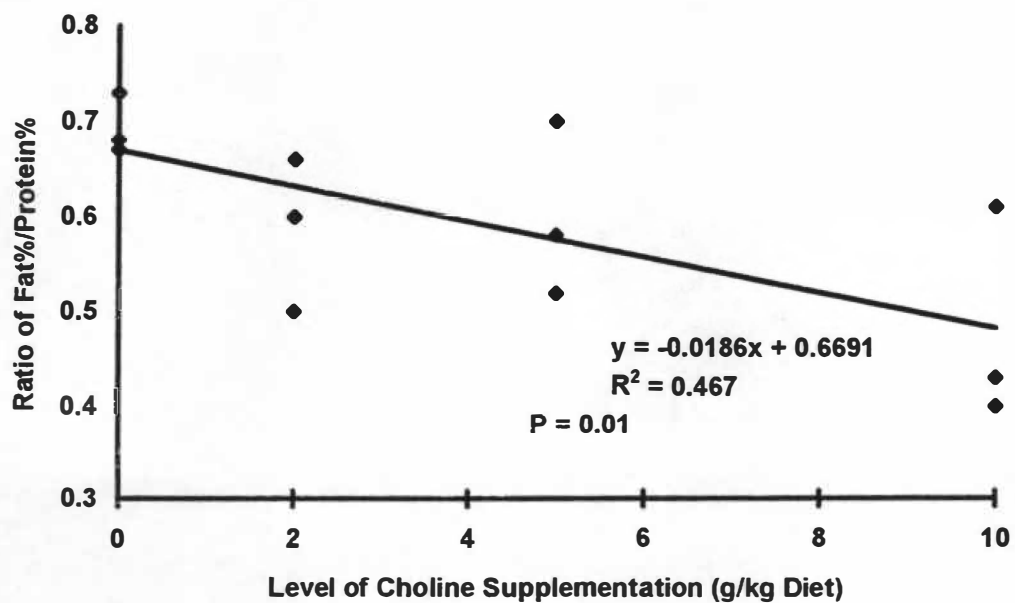
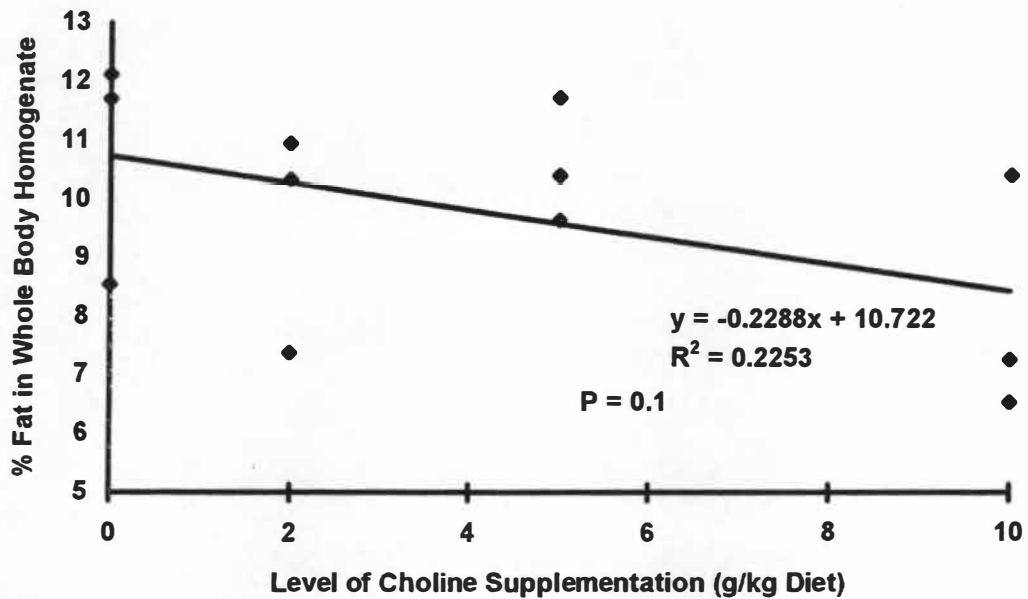


Figure 4-2: Regression analysis of the effect of varied levels of choline supplementation on % body fat (top) and the ratio of %fat / %protein (bottom) in whole body homogenates of guinea pigs.

Total body carnitine was estimated by assaying the carcass carnitine and adding back the carnitine from the tissues that were removed (**Table 4-9**). When expressed on a per kg body weight basis the carnitine concentration was higher in the choline supplemented animals, with the difference being significant in 2 of the 3 choline supplemented groups. When expressed on $\mu\text{mol}/\text{animal}$ basis the differences were not significant, probably due to the large variation in animal size between the two sets.

Carnitine excretion was estimated for the study period by averaging the daily excretion and assuming that average to be typical for all days during the study. Carnitine conservation was then estimated for the study period by subtracting the average amount excreted in the choline supplemented animals from that excreted by the control animals. This was compared to the average increase in total body carnitine for each choline supplemented group over the unsupplemented group (**Table 4-9**). The increased total body carnitine accounted for approximately 100% of the carnitine conserved in the animals supplemented with 2 and 10 g choline per kg diet, and about 40% of the conserved carnitine in the group supplemented with 5 g per kg diet.

The d 21 urine and plasma collections were used to further describe the effects of choline on renal handling of carnitine (**Table 4-10**). Creatinine excretion and clearance were not affected by choline supplementation. Carnitine clearance was reduced in choline supplemented groups but only significantly in the 2 g choline/kg diet group. Fractional tubular reabsorption was significantly increased in all of the choline supplemented groups.

Table 4-9
Total Body Carnitine and Urinary Conservation of Carnitine
in Guinea Pigs Supplemented with Varied Levels of Choline for 21 Days¹

	Level of Choline Supplement (g/kg diet)			
	0	2	5	10
Total Carnitine				
Carcass ($\mu\text{mol/kg bw}$)	372.5 ± 39.5^a	494.5 ± 22.0^b	403.8 ± 20.9^a	481.3 ± 23.1^b
Carcass ($\mu\text{mol/animal}$)	154.4 ± 27.9	220.7 ± 41.9	179.9 ± 37.8	213.5 ± 58.0
S. Intestine ($\mu\text{mol/animal}$)	1.3 ± 0.1	1.4 ± 0.2	1.7 ± 0.2	1.9 ± 0.2
Heart ($\mu\text{mol/animal}$)	2.7 ± 0.3	3.5 ± 0.5	3.8 ± 0.7	2.6 ± 0.4
Brain ($\mu\text{mol/animal}$)	0.7 ± 0.2	1.0 ± 0.2	0.8 ± 0.1	0.9 ± 0.2
Liver ($\mu\text{mol/animal}$)	7.2 ± 1.3	7.8 ± 2.0	8.7 ± 2.4	8.4 ± 2.8
Kidney ($\mu\text{mol/animal}$)	2.2 ± 0.5	2.2 ± 0.4	2.2 ± 0.3	2.2 ± 0.4
Muscle ($\mu\text{mol/animal}$)	1.4 ± 0.1	2.01 ± 0.2	1.7 ± 0.2	1.7 ± 0.2
Total Body Carnitine				
($\mu\text{mol/animal}$)	169.8 ± 29.7	238.8 ± 44.9	198.7 ± 41.3	231.3 ± 61.8
Estimated Carnitine Excretion During				
the Study ($\mu\text{mol/animal}$)	126.8 ± 28.8	62.6 ± 23.0	55.4 ± 13.8	64.7 ± 17.1
Estimated Carnitine Conservation				
($\mu\text{mol/animal}$)		64.3	71.4	62.1
Average Increase in Total Body				
Carnitine ($\mu\text{mol/animal}$)		69.0	28.9	61.5

Total body carnitine was determined by adding total carnitine in each tissue removed to the total carcass carnitine for each animal. Carnitine excretion during the study was estimated by averaging the 24h excretions during the experimental period and multiplying by 21d. Carnitine conservation was estimated by subtracting carnitine excretion during the experimental period in choline supplemented animals from that of unsupplemented animals. All values are the group mean \pm SEM except for some estimates, for which only the values are shown.

Different superscripts indicate significant differences by ANOVA and Tukey's test ($P < 0.05$, $n = 5$).

Table 4-10
Effects of Varied Levels of Choline Supplementation on Renal Excretion
and Reabsorption of Carnitine and Creatinine¹

	Level of Choline Supplement (g/kg diet)			
	0	2	5	10
Creatinine Excretion ($\mu\text{mol/d}$)	110.4 ± 15.2	78.4 ± 17.4	110.8 ± 22.1	108.4 ± 25.6
Creatinine Clearance (mL/minute)	0.08 ± 0.03	0.06 ± 0.02	0.11 ± 0.02	0.09 ± 0.04
Carnitine Clearance (mL/minute)	0.005 ± 0.002^a	0.001 ± 0.001^b	0.003 ± 0.001^{ab}	0.003 ± 0.002^{ab}
Fractional Tubular Reabsorption (%)	92.14 ± 1.94^a	97.92 ± 0.29^b	97.3 ± 0.25^b	98.16 ± 0.55^b

¹Creatinine and carnitine clearances were calculated using the equation: Clearance = $(U_x/P_x)V$ where U = urinary excretion of x, P = plasma concentration of x, x = creatinine or carnitine, and V = urine flow in ml per minute. Fractional tubular reabsorption was calculated by the formula:

$$\text{FTR}(\%) = \{1 - (\text{Ucne}/\text{Pcne}) (\text{Pcreat}/\text{Ucreat})\} \times 100$$

Where FTR = fractional tubular reabsorption, Ucne = urinary excretion of carnitine $\mu\text{mol/d}$, Pcne = plasma concentration of carnitine ($\mu\text{mol/L}$), Pcreat = plasma concentration of creatinine ($\mu\text{mol/L}$), and Ucreat = urinary excretion of creatinine ($\mu\text{mol/d}$). All values are shown as the group mean \pm SEM. Values with different superscripts are significantly different by ANOVA and Tukey's test ($P < 0.05$, $n = 5$).

Discussion

The first set of animals maintained their weight during the study, but did not gain weight, because they had reached adult weight at the onset of the study. The second set, on the other hand, gained 100-150 grams during the study. This resulted in a near zero feed efficiency in the adult animals, which would be expected in animals that are now growing. The feed efficiency the younger animals varied from a low of 0.19 (g body weight/g feed) in the nonsupplemented group to a high of 0.21 in the animals supplemented with 10g choline per kg diet. Neither feed efficiency, beginning body weight, or final body weight were significantly different among groups.

Urine and Plasma Carnitines

Carnitine excretion, in urine, was not significantly affected by choline after 2 days of choline supplemented diet. The excretion in the groups supplemented with 5 and 10 g choline per kg diet was about half that of the nonsupplemented group, but the carnitine excretion on day two in the group supplemented with 2 g choline per kg diet was nearly identical to that of the control animals, suggesting a delayed response at the lower dose. By day 5 of the study, there was a significantly lower carnitine excretion in all of the choline supplemented guinea pigs, a trend that continued for the remainder of the study. Analysis of variance also revealed a significant increase over time in the urinary excretion of total carnitine and the NEC and AIAC fractions. The

time dependent increase in urinary carnitine excretion were prevented by choline supplementation, and total carnitine excretion was significantly lower on day 5 than day 0 in the highest supplemented group. In previous studies there was a significant increase in plasma carnitine concentration in guinea pigs. In this study, plasma carnitine was not significantly higher in the choline supplemented groups. This may be due to the difference in the statistical tests and number of animals. In the previous studies (Daily and Sachan 1995) there were only two groups with an n of 12 and significance determined by a t-test. In this study there were four groups of 5 animals each and significance was determined by ANOVA and Tukey's test (a more conservative test than a t-test) (Table 4-6). When the plasma data is analyzed using Student-Newman-Keuls test, the difference between the nonsupplemented and 2 g choline/kg diet groups is significant.

Tissue and Intestinal Carnitine

Skeletal muscle carnitine concentrations were higher in choline supplemented animals and was significant in the groups supplemented with 2 and 10 g choline per kg diet. There was an apparent dose effect, with the highest carnitine concentration in the muscle of the guinea pigs receiving the highest choline supplement. The higher muscle carnitine in choline supplemented animals is consistent with the previous studies. The muscle carnitine in the group supplemented with 10 g choline per kg diet was 129% of that in the nonsupplemented group. Muscle carnitine has been shown to

increase 10% in humans supplemented with 1g/day of carnitine for 6 months (Arenas et al 1991),

There was no indication of an intestinal carnitine loss in choline supplemented guinea pigs. It was postulated that if carnitine was being lost from the intestinal tissue, and not reabsorbed, this would be apparent as a decreased tissue concentration. Instead, there was a dose-dependent increase in carnitine concentration in small intestine tissue. This suggested that intestinal tissue is affected by choline supplementation, in a manner similar to muscle tissue. It was also postulated that if carnitine was being lost into the lumen, then lumen carnitine content of the choline supplemented animals would be higher. The reverse turned out to be true, the lumen carnitine content was highest in the control animals, although none of the differences were significant. The carnitine content of feces were also not different. The results do not support the hypothesis that carnitine was being lost from the intestine or that such loss resulted in a compensatory decrease in urinary carnitine excretion. On the contrary, there was an accretion of carnitine in the intestinal tissue of choline supplemented animals.

Body Composition

The body composition data was complicated by the age difference in the two sets of guinea pigs (Table 4-8). In the older animals, fat was approximately 24% of the body mass, but in the younger animals only about 16%; and age had a much greater

affect on body fat than did choline supplementation. Nevertheless, nonsupplemented animals in both groups had a higher percentage of body fat and a higher fat/protein ratio than did the choline supplemented animals, but this was only significant for those supplemented with 10 g choline/kg diet in the second set of younger animals. The otherwise lack of significance is not surprising because of the high variation due to age effect when both sets were combined, and the low numbers per group ($n = 2$ and $n = 3$) when the sets were considered individually. A regression analysis (Figure 4-2), however, indicated no difference in fat percentages with increasing choline supplementation ($P > 0.05$), but a significant dose-dependent effect of choline in reducing the ratio of fat/protein ($P = 0.01$) in the younger animals. There was no effect of choline on body composition in the mature animals. While the evidence for an effect of choline supplementation in reducing body fat is intriguing, it is not strong enough at this point to make a final conclusion.

The total body carnitine was approximately 29% higher, on a per kg body weight basis, in choline supplemented animals, confirming that the reduced carnitine excretion represents a choline induced carnitine conservation (Table 4-9). The estimates of conservation of urinary carnitine closely matched the increase in total body carnitine.

Others have demonstrated that choline deficiency can cause a carnitine deficiency (Tsai et al. 1974) with reduced liver, muscle, and whole body carnitine. It has also been shown that carnitine deficiency resulting from choline deficiency impairs fatty

acid oxidation (Corredor et al 1967). The unique aspect of this line of research is that choline supplementation, in choline sufficient humans and animals, increases tissue concentration and total body stores of carnitine.

Renal Regulation of Carnitine

The mechanism of the choline-carnitine interaction is unknown. The two best characterized aspects of this interaction are the decrease in carnitine excretion and increase in tissue accumulation of carnitine in choline supplemented animals. The decreased carnitine excretion is not a result of impaired kidney function since creatinine excretion and clearance rates are not affected in choline supplemented animals (Table 4-10). Instead, the decreased urinary excretion of carnitine is a result of an increased fractional tubular reabsorption of carnitine in the kidney. There is no easy explanation for how choline causes an increase in tubular reabsorption of carnitine. Three possible mechanisms may account for the choline induced increase in renal carnitine reabsorption. 1) Choline acts as a co-transport molecule that facilitates the reabsorption of carnitine. 2) Choline reabsorption rate is increased and that a lack of specificity allows carnitine, a structural analog of choline, to also be reabsorbed by the same mechanism. 3) The renal carnitine transport protein may be upregulated following choline supplementation, causing an increase in fractional tubular reabsorption.

The first possibility, that choline may act as a co-transport molecule seems unlikely. Choline and carnitine appear to use different transport mechanisms, since carnitine transport is a sodium linked process (Stadler et al. 1993) and choline is not (Zeisel 1981). Furthermore, in studies of carnitine transport in rat renal brush-border membrane vesicles, choline has been shown to have no effect on carnitine transport at physiological concentrations. At very high concentrations of choline (100 mmol/L), carnitine transport was inhibited by more than 50% (Stieger et al. 1995). There is, therefore, little reason to suspect that a co-transport mechanism is responsible for the increase in renal reabsorption of carnitine in choline supplemented humans or animals.

The second possibility, that choline supplementation might upregulate choline reabsorptive mechanisms is counter to what would be expected in this situation. Upregulation of choline reabsorption would be more likely in choline deficiency. There is some evidence for cross-reactivity between the two molecules, however. Carnitine-acetyltransferase also has weak choline acetyltransferase activity, but choline-acetyltransferase is not cross reactive (Sastry & Janson 1994). This type of interaction is suspect because of the similar structure of choline and carnitine, but no evidence is available to support such a conclusion.

The third possibility, that the transport mechanism for carnitine is upregulated, is the most plausible. Although not yet identified, there is strong evidence for the existence of a carnitine transport protein. Berardi et al (1995) expressed rat kidney mRNA in *Xenopus laevis* oocytes, which induced an increased sodium dependent

carnitine transport in the oocytes. There is also a well characterized inheritable defect in the juvenile visceral steatosis (*jvs*) mouse involving renal reabsorption of carnitine. This condition is a autosomally inherited recessive defect that, in homozygotes, results in a depletion of serum, liver, and muscle carnitine (Hayakawa et al. 1990, Koizumi et al. 1988). Horiuchi et al (1994) demonstrated that the primary defect in the *jvs* mouse is in the renal reabsorption system for carnitine. It has been shown that the administration of large amounts of carnitine can correct the consequences of the defect (Horiuchi et al. 1992, Hotta et al., 1996, Uenka et al. 1996). As of now, the putative carnitine transport protein has not been identified, antibodies to it have not been produced, nor the gene cloned; therefore the study of its expression is not possible. The possibility that choline may in some manner increase the expression of this protein, however, in an attractive theory that awaits further study.

Conclusions

There was not a clear dose response in this study. For most measured parameters there were no significant differences between the lowest and highest supplemental doses. Only the intestinal tissue data and regression of %fat/%protein in the carcasses were dose dependent. This study has demonstrated, therefore, that low doses of choline, have a near maximal effect on carnitine conservation in guinea pigs.

The optimal dose, with regard to specific tissues, is still not clear since the effect was greatest in skeletal muscle at the higher dosages, but not in heart.

This study also suggests that the cause of the choline induced decrease in urinary excretion of carnitine is, in part, related to an increase in the renal fractional tubular reabsorption of carnitine; and that there is not an intestinal loss of carnitine. Finally, the lower %fat/%protein ratio in choline supplemented guinea pigs suggests a metabolic consequence of the choline-carnitine interaction. Other functional consequences of choline supplementation were investigated in the next study.

Chapter 5

Metabolic Consequences of Choline-Carnitine

Interactions

Introduction

The decreased %fat/% protein ratio of the choline supplemented guinea pigs in the dose response study in chapter 4 suggested that choline induced carnitine accretion in tissues might have metabolic consequences. Carnitine supplementation has been utilized in attempts to improve athletic performance in several studies (see **Table 5-1**). Two primary issues remain unresolved from these studies. 1) Does carnitine supplementation increase muscle carnitine concentrations? 2) If muscle carnitine is increased, is the capacity for the muscle to oxidize fatty acids also increased?

Severe carnitine depletion unquestionably results in a decreased capacity for fatty acid oxidation. Muscle fatigue and weakness is a classic symptom of carnitine deficiency (Worthily 1983, Anonymous 1985). Valproic acid is a anticonvulsant drug know to cause carnitine deficiency (Isom 1984). The effect of valproic acid therapy, with and without supplemental carnitine, on the oxidation of energy substrates

Table 5-1
Review of Carnitine Supplementation and Exercise

Reference (species)	Carnitine Dosage g/d	Duration days	Results
Arenas et al. (1991) (humans)	1	180	Muscle carnitine increased over placebo treated controls
Vukovich et al. (1994) (humans)	9	14	No effect on muscle carnitine or fat oxidation
Gorostiaga et al. (1989) (humans)	2	28	RQ decreased during exercise in carnitine vs placebo supplemented
Wyss et al. (1989) (humans)	3	25	RQ decreased during exercise in carnitine vs placebo supplemented
Decombaz et al (1993) (humans)	3	7	No effect on RQ or on work capacity.
Veechiet et al (1990) (humans)	2	1h prior to exercise	Increased work capacity but no effect on RQ
Heinonen and Takala (1994) (rats)	Carnitine depleted to 48% of controls		No effect on work capacity or fat oxidation

has been studied in humans (Melegh 1994). In this study 8 patients receiving valproic acid therapy had an average resting non protein RQ of 0.93, indicating that carbohydrate was the predominant energy source. With supplemental carnitine, the resting RQ decreased to 0.85, a more typical RQ which indicates that fat and carbohydrates are contributing about equally to energy demands.

There are conflicting studies on the effects of carnitine supplementation on energy substrate utilization in normal individuals. Vukovich et al (1994) reported that carnitine supplementation (9g/d) for 2 weeks had no effect on either exercise capacity, respiratory exchange ratio (RER) during 1h of submaximal exercise at 70% $\text{VO}_{2\text{max}}$, or muscle carnitine concentrations. However, Arenas et al. (1991) demonstrated very different effects of carnitine supplementation in a 180 d placebo controlled experiment. Exercise training (long-distance running) significantly decreased the muscle concentration of carnitine (28.0 - 26.3 $\mu\text{mol/g}$ noncollagenous protein) in subjects given a placebo, but subjects given 1 g/d of carnitine had a significant increase in muscle carnitine (26.3 - 29.0 $\mu\text{mol/g}$ noncollagenous protein) while participating in the same exercise routine. A similar effect was seen in sprinters. The different effects on muscle carnitine between the two studies may be an indication that supplementation with smaller doses (1 vs 9 g) over a long period of time (180 vs 7 d) is more effective than higher doses for a short time. Unfortunately, Arenas et al. did not determine a functional effect by measuring RER, endurance, or other possible indicators of changes in oxidative capacity. Other researchers have, however,

reported an effect of carnitine supplementation on exercise capacity and RER, but did not determine effects on muscle carnitine concentrations (Table 5-1).

One recent study (Heineken and Tackle 1994) found no effect of “moderate” carnitine depletion in young rats on exercise capacity. In this study, the control group was fed a commercial pellet diet, while the carnitine devoid group was given a carnitine free isocaloric liquid diet. The carnitine deficient animals were given free access to their diet, and the control group paired with one of the carnitine devoid animals, and food adjusted so that each rat received the same amount of nutrients, except carnitine, in each pair. The muscle carnitine concentration in the carnitine devoid animals was 48% of that in the control animals. The lower muscle carnitine, however, did not result in a reduced swimming time to exhaustion, or in a reduced oxidation of ^{14}C palmitate after an 8h fast. The authors concluded that moderate carnitine depletion does not affect exercise capacity or fatty acid oxidation. However, there were several problems with this study. First the carnitine assay measured only free and short-chain acyl carnitines. Long-chain acyl carnitines were not determined. If there was a higher percentage of long-chain acyl carnitine in the devoid group, then the differences in muscle carnitine content would be correspondingly smaller. Second, these were young growing rats (same age as the second set of guinea pigs in the dose response study), if carnitine sufficiency resulted in a comparatively lower fat/lean body mass, as seen with choline supplemented guinea pigs, then buoyancy would be affected and could have a significant impact on swimming time. That is, if the

carnitine devoid rats had a higher % body fat, they would be more buoyant and require less work to swim. There was no body composition analysis in this study, although the carnitine deficient rats gained significantly less weight during the feeding period. The results of this study are difficult to interpret, and the validity of the author's conclusions is not possible to access without additional information.

We have demonstrated that choline supplementation increases muscle carnitine (Daily and Sachan 1995) and carcass carnitine (Table 4-9) in normally fed guinea pigs. However we have not clearly demonstrated a functional consequence for this increase in muscle carnitine. The objective of this research, therefore, was to determine if choline induced carnitine accretion in muscle increases fatty acid oxidative capacity and endurance in guinea pigs using indirect calorimetry under normal, starvation, and exercise conditions.

Materials and Methods

The protocol for this research was approved by the Animals Care and Use Committee of the University of Tennessee. Twenty male Hartley guinea pigs (SASCO, Omaha, NE) were divided into 2 groups to be fed ground commercial diet (Purina Guinea Pig Chow no. 5025, Purina Mills, Richmond, IN) with or without supplemental choline (3g/kg diet from choline chloride). It was not possible, to study more than two animals simultaneously, therefore, animals were ordered in pairs and

continued through the study as pairs, so that there would not be differences in age (Age = 9.7 ± 1.2 weeks for both groups), and minimal differences in body weight between groups. The animals that began as pairs for the starvation study were then used in the exercise study. Animal diets, and weights are compared in **Table 5-2**.

Experimental Design - Fasting Study

This study was designed to monitor gas exchange in all animals over an 8h period prior to beginning the experimental diets under fed and unfed conditions and again after 2 weeks on the experimental diets. When animals were monitored in the unfed state, food was provided at 800 hours and removed at 900 hours. At 900 hours, gas measurements were begun and each animal was measured during each one hour period for 8 hours. When measurements were made during fed conditions, the animals had free access to food except when in the gas monitoring chamber. The following schedule was followed for each pair of guinea pigs.

- Week 1 - Animals monitored for gas exchange twice for 8h under fed conditions. Urine was collected for 24 h following the gas measurements with animals having free access to food.
- Week 2 - Animals monitored once for 8h with food withheld for the entire time. Urine was collected for 24 h following the gas measurements while animals still unfed. Animals were, therefore, unfed for 8 h prior to beginning the urine collection and 32 h at the end of the collection.
- Week 2 - Following unfed experiment, animals were placed on experimental diets.
- Week 3 - Animals on experimental diets.
- Week 4 - Animals on experimental diets
- Week 5 - Animals monitored for 8h under fed conditions, as before.
- Week 6 - Animals monitored for 8h under unfed conditions as before.

Table 5-2
Choline Content of Guinea Pig Diets and Initial & Final Body Weights¹

	Experimental Group (Level of Choline Supplement)	
	0	3
Diets		
Choline Supplement (g/kg)	0	3.0
Endogenous Choline (g/kg)	1.85	1.85
Total Choline (g/kg)	1.85	4.85
Animal Weights - Starvation Study		
Initial Body Weight	524.8 \pm 13.0	502.9 \pm 15.4
Final Body Weight	686.1 \pm 22.0	667.2 \pm 23.1
Animal Weights - Treadmill Study		
Initial Body Weight	972.6 \pm 53.7	938.8 \pm 25.6
Final Body Weight	1025.3 \pm 39.2	944.9 \pm 28.1

¹Diets were prepared by grinding commercial diet and adding either no supplement or choline from choline chloride. Values are means for the group \pm SEM for body weights. The same diets were continued through the starvation study and the treadmill study.

Animals were fed each morning 1h prior to beginning the experiment. On days when food was withheld, animals were fed normally in the morning but food was removed when each animal was placed in the metabolic chamber. During each 1h period, animals were placed in the sealed metabolic chamber for 20min and gases collected in a gas collection bag (allowed to fill for 1min) after 10, 15, & 20min. The O₂ and CO₂ content of each gas collection was determined using an Ametek S-3A/II oxygen analyzer and Ametek CD-3A carbon dioxide analyzer, values for the 3 collections were averaged and the average used as the value for that 1h period.

Fed-state measurements were made twice during pre-supplement period and experimental diet period. There was only one day of unfed measurements for each dietary period, because of the possibility that the animals might not fully recover from being unfed more times. After each 8h gas measurement experiment, the guinea pigs were placed in metabolic cages (not the metabolic chamber that was used for calorimetry) for 24h urine collections. Either feeding or unfeeding, whichever was used in the gas measurements, was continued for the 24h urine collection. Urine was collected and frozen at -20°C for analysis of carnitine and urea nitrogen at a later time.

Experimental Design - Treadmill Study

The treadmill study was designed to evaluate choline induced changes in RER, VO₂, and endurance during submaximal exercise. All animals were trained to run on

treadmill prior to starting the experiments. The average age of the pairs of guinea pigs was $8.1 \pm$ months, and the average time on experimental diets was 5.5 ± 0.9 months. Each pair was trained on the treadmill for the same length of time and at the same intensity to insure that no differences in training effects between groups influenced the outcome. Treadmill training sessions were kept to 10 minutes to minimize any training effect, since the purpose of this study was to evaluate the effect of supplemental choline and not training.

There were four time periods in the experimental protocol, early morning, late morning, early afternoon, and late afternoon, approximately 0900, 1100, 1300, 1500 hours respectively, depending on how long the preceding animals ran. Each animal was rotated between time periods and performed 4 running bouts each (except 1 which refused to run after two bouts).

The treadmill was set at a 5% incline with a speed of 15m/min. This incline and speed was determined during trial runs to be equivalent to a “fast walk”, and an exercise work rate that could be tolerated for extended periods. Animals were placed in the treadmill unit for 15min prior to commencement of running. Air flow through the unit was set at 2.1 L/min. After 15min the first gas collection was taken and analyzed as in the starvation study, and the first collection designated time 0. Immediately following the first gas collection the treadmill was started, air flow increased to 3.8 L/min, and gas collected and analyzed at 5 min intervals until the end of the experiment. Animals continued running for 2h or until they refused to run

longer, preferring the electric shock to running further. All animals were given a minimum of 3 days recovery between exercise experiments.

After completion of the treadmill studies the animals were allowed to remain on their respective diets for a minimum of 2 weeks before being killed. Four animals at a time were placed in metabolic cages with or without food as follows: 1 nonsupplemented-fed, 1 nonsupplemented-unfed, 1 choline supplemented-fed, 1 choline supplemented-starved, and urine was collected for 24h. After the 24h urine collection the animals were anesthetized with methoxyflurane, blood taken by cardiac puncture, and the animals killed by decapitation. Heart, liver, brain, kidney, and muscle were collected for carnitine assay and frozen at -80°C . Liver and muscle tissue were also processed for the CPT assay which followed killing of the animals.

Gas Analysis

Gas analysis instruments determine the percentage of the measured gases in an air sample. In this case O_2 and CO_2 are determined and N_2 assumed to be the remainder, a reasonable assumption (Bursztein et al. 1989). O_2 and CO_2 are determined using the following equations.

$$\text{VO}_2 = (\text{VI} \times \text{FIO}_2) - (\text{VE} \times \text{FEO}_2)$$

Where: VO_2 = volume of oxygen utilized.

FIO_2 = Fraction of oxygen entering the chamber (0.2093 for room air).

FEO_2 = Fraction of oxygen exiting the chamber.

VE and VI = the air flow in and out of the chamber and are assumed to be equal.

$$VCO_2 = (VE \times FECO_2) - (VI \times FICO_2)$$

Where: VCO_2 = volume of carbon dioxide produced.

$FECO_2$ = fraction of carbon dioxide exiting the chamber.

$FICO_2$ = fraction of carbon dioxide entering the chamber (0.0003 for room air).

VE and VI = air flow in and out of the chamber and are assumed to be equal.

These equations, therefore, use the fraction of the gases entering and leaving the chamber, to determine the change in gas concentration from room air. The decrease in the fraction of O_2 and the increase in the fraction of CO_2 are both multiplied by the air flow per minute through the chamber to give the volume of each gas utilized or produced. There are two adjustments made to the basic equations above that are used in actual practice.

First VE and VI are adjusted to their equivalents at standard temperature and pressure (STPD) from the values obtained at ambient temperature (ATPS) using the following equation for the conversion factor (Bursztein et al. 1989, Osborn et al. 1969):

$$K1 = \frac{273 + 37^\circ C}{273 + T^\circ A} \times \frac{BP - PAH_2O}{BP - PH_2O(37^\circ C)}$$

where:

$K1$ = conversion factor for converting gas volume at ATPS to STPD

273 = the absolute temperature (in degrees Kelvin) at $0^\circ C$

BP = barometric pressure

$T^\circ A$ = the ambient temperature at which the measurements were made (degrees C)

PAH_2O = the pressure of water vapor at the ambient temperature

PH_2O = the pressure of water vapor.

There is an assumption in the basic equations that because N_2 is not absorbed, it remains constant in inspired and expired air. In actual practice there is usually a small difference in the percentage of N_2 between the two, and this is the second adjustment that is made to the basic equations. For the concentration of nitrogen to be the same in both inspired and expired air, the volume of CO_2 added to the expired gases must be equal to the volume of O_2 removed, in other words, there must be an RER of 1. This not usually the case. Therefore, the following equations are expanded versions of the basic equation, and are used to adjust for this effect in calculating VO_2 and VCO_2 .

$$VCO_2 = \left\{ FECO_2 \left[\frac{1 - FECO_2 - FEO_2}{0.7904} \right] - FICO_2 \right\} \times AF$$

$$VO_2 = AF \times \left\{ FIO_2 - \left[FEO_2 \times \frac{1 - FECO_2 - FEO_2}{0.7904} \right] \right\}$$

Where:

AF = air flow through the chamber

0.7904 = fraction of N_2 in room air

VCO_2 = volume of carbon dioxide produced.

$FECO_2$ = fraction of carbon dioxide exiting the chamber.

$FICO_2$ = fraction of carbon dioxide entering the chamber (0.0003 for room air).

VO_2 = volume of oxygen utilized.

FIO_2 = Fraction of oxygen entering the chamber (0.2093 for room air).

FEO_2 = Fraction of oxygen exiting the chamber.

A step by step description of the indirect calorimetry measurements is available in appendix 2.

Carnitine and Urea Nitrogen Assays

Carnitine was assayed using the radioenzymatic assay described in the materials and methods section of chapter 4 (Cederblad and Lindstedt 1972, Sachan et al 1984). Urea nitrogen was determined in the urine samples using Sigma kit no. 640, a spectrophotometric urease/Berthelot determination for serum, plasma, or urine. In this assay urea is first hydrolyzed releasing ammonia. The ammonia reacts with alkaline hypochlorite and phenol in the presence of sodium nitroprusside (a catalyst) to form indophenol. The concentration of ammonia is directly proportional to the absorbance of indophenol at 570 nm, and the concentration is calculated based on the absorption of a standard solution of urea (10.7 mmol/L or 30 mg/dl).

Carnitine Palmitoyltransferase -I Assay

CPT-I was assayed in intact mitochondria from muscle and liver processed immediately following removal from the animals. Mitochondria were prepared according the method of Johnson and Lardy (1967). The tissues were first minced by cutting with scissors in cold 0.25 mol/L sucrose and then homogenized using a Potter-Elvehjem homogenizer with a teflon pestle rotating at 125 rpm. For liver, 3 passes on the homogenizer was used for the homogenization, additional passes were required for homogenization of muscle. The homogenate was then centrifuged at 600

x g for 10 minutes and the supernatant containing the mitochondria decanted into a second set of centrifuge tubes. The mitochondria were then centrifuged at 12,000 x g for 10 minutes, the pellet was then resuspended in 0.25 mol/L sucrose and centrifuged again at 12,000 x g for 10 minutes. The pellet was resuspended as before, protein determined by biuret assay and the mitochondrial suspension adjusted to 10mg protein/ml.

The method of Bremer (1981) was used to estimate CPT-I activity in muscle and liver mitochondria. Each assay mixture contained in a total volume of 0.5 ml: 82 mol/L sucrose, 70 mol/L KCl, 1 mol/L EGTA, 70 mol/L imidazole, 1 µg antimycin A, 2 mg BSA, 0.5 mol/L L-carnitine (0.4 mCi/mmol of L-[methyl-³H] carnitine) and 40 µmol/L palmitoyl CoA. For determination of malonyl CoA inhibition, malonyl CoA concentrations were 0, 10, 25, and 50 µmol/L. For determination of the apparent k_m of palmitoyl CoA, concentrations were 10, 50, 100, and 200 µmol/L and for the determination of k_m for carnitine, concentrations were 0.1, 0.5, 1, and 2 mmol/L.

Inhibition data was plotted in a double reciprocal manner : 1/ %inhibition on the y axis and 1/concentration of malonyl CoA on the x axis. The reciprocal of the inhibitor concentration that gives 1/2 of maximum inhibition ($1/I_{50}$) is at the intersection of the x axis and the reciprocal of the maximum inhibition ($1/I_{max}$) is at the intersection of the y axis. The calculations were performed with the assistance of the Hyperbolic Regression Analysis 1.01 software (J.S. Easterby, Liverpool, UK). More details for the assay are available in the protocol in appendix 1.

Statistics

The urinary excretion of carnitine in fed and unfed states was analyzed using a split plot, repeated measures ANOVA using the MIXED procedure of SAS (Cary, N.C.). When the F test indicated differences between groups, the differences were separated using Fisher's protected least significant difference test. Tissue carnitine concentrations, urinary excretion and plasma concentrations of carnitine, and CPT activity were compared using ANOVA and Student-Newman-Keuls test when the ANOVA indicated differences among groups. Running time between groups was compared using Student's t test.

Results

Fasting Study

There was no effect of choline supplementation on either RER or oxygen consumption when animals were fed or when food was withheld over 8h. The 8h starvation did affect RER in both groups with the RER decreasing over time from about 0.95 to about 0.79 (**Figures 5-1 & 5-2**). There was an apparent effect of

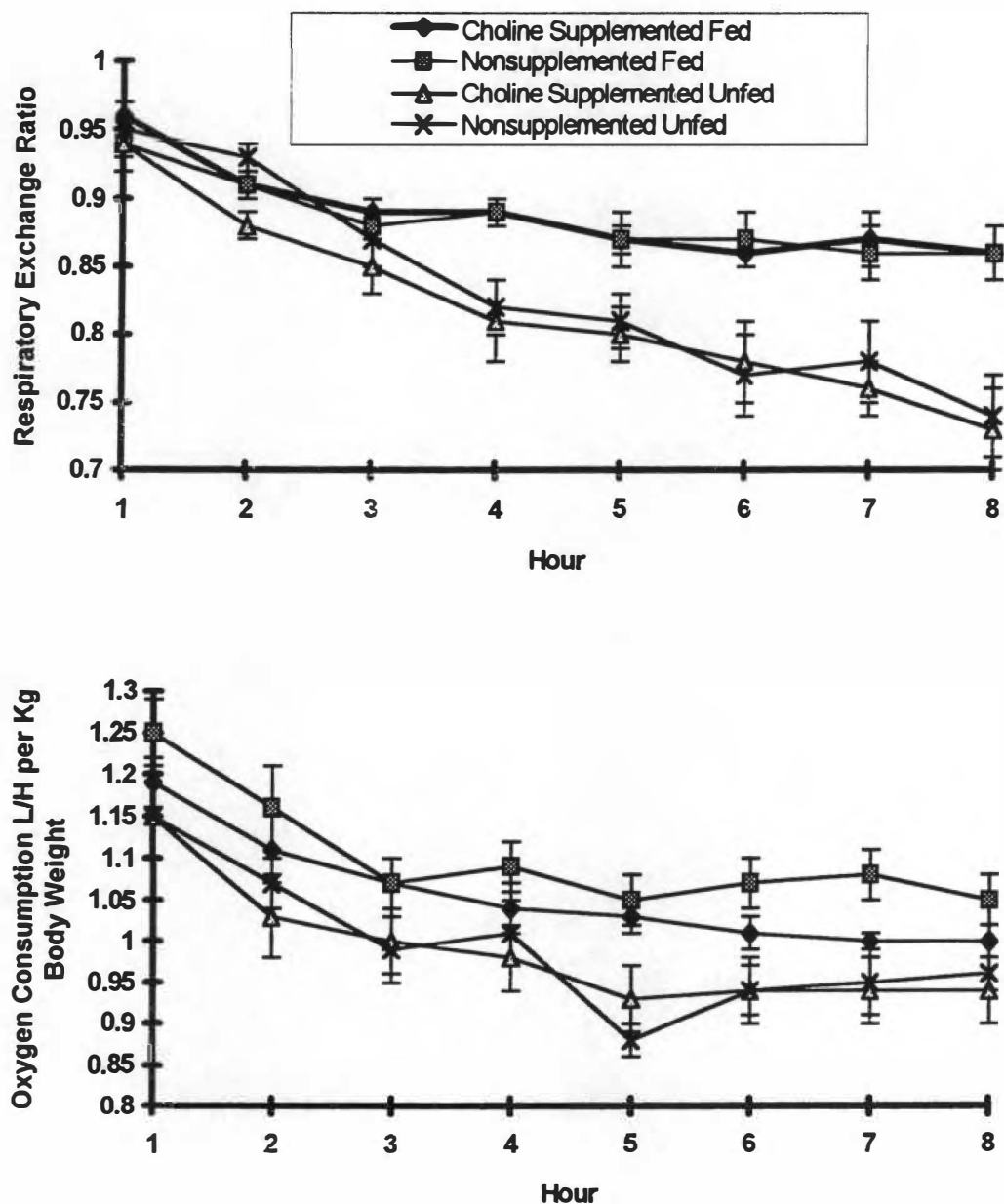


Figure 5-1: Pre-experimental diet respiratory exchange ratios and oxygen consumption. These pre-experimental diet measurements compared animals that would in be either choline supplemented or nonsupplemented groups after starting the experimental diets. Values are means for the group \pm SEM (n = 10 when food withheld, n = 20 when fed).

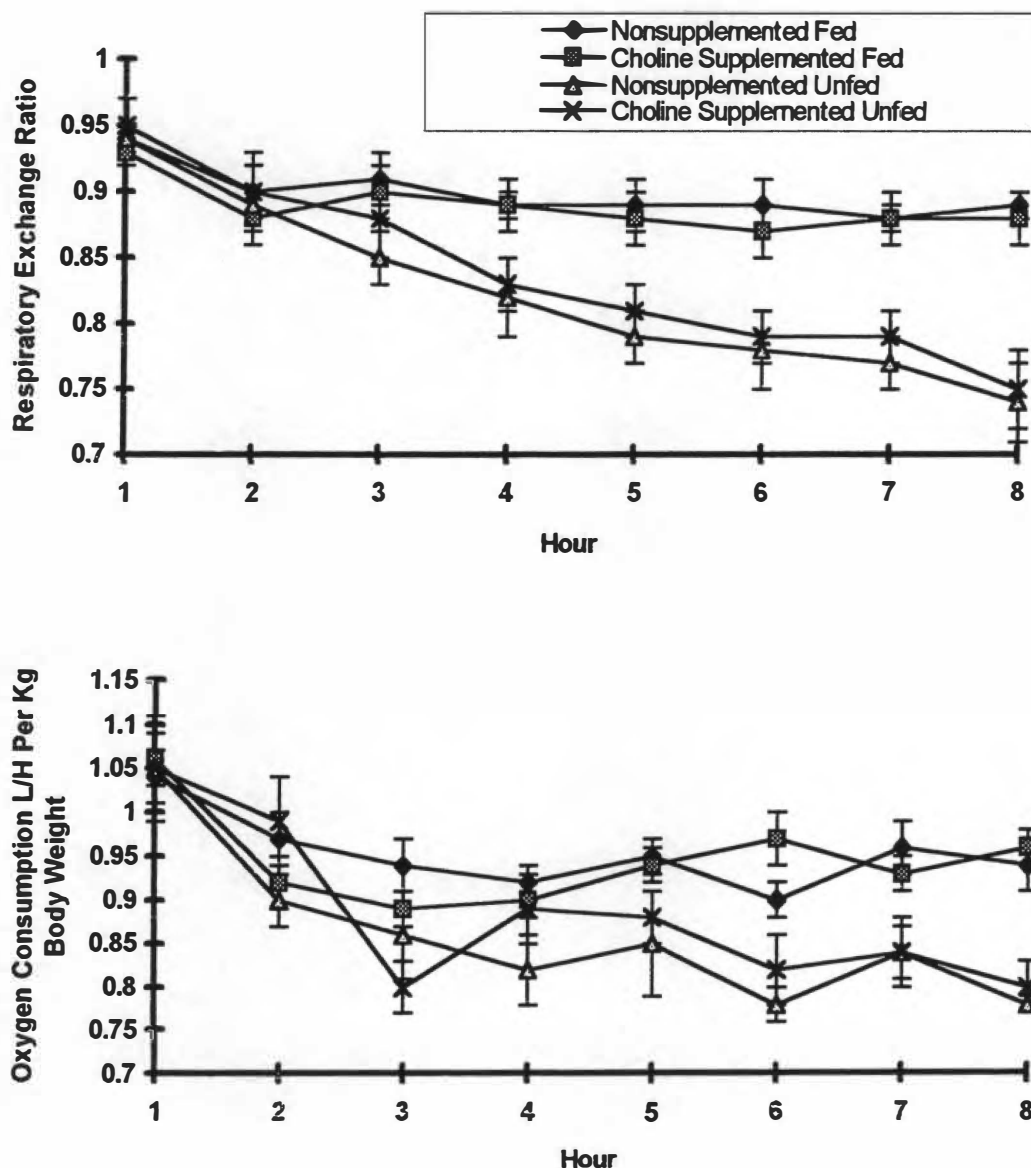


Figure 5-2: Post-experimental diet respiratory exchange ratios and oxygen consumption. The animals were either choline supplemented (ChS) or non-supplemented (NS). Values are means for the group \pm SEM. There were no differences between NS and ChS groups but RER and oxygen consumption were significantly lower after 4h & 6h respectively, when food was withheld as compared to fed states, by ANOVA and Student-Newman-Keuls test ($P \leq 0.05$, $n = 10$ when food withheld, $n = 20$ when fed).

being unfed on oxygen consumption, with oxygen consumption decreasing more over time when food was withheld.

There were no differences between groups in 24h urinary excretion of carnitine prior to starting the experimental diets. There was, however, an effect of starvation with both groups excreting less NEC but more ASAC and AIAC. ASAC was increased nearly 10 fold and total carnitine excretion almost 3 fold as a result of starvation (**Table 5-3**). There was no significant effect of choline supplementation on urinary excretion in this study (Table 5-3). Urinary excretion of acyl and total carnitines was increased during starvation in both groups. There were also no significant differences in 24h nitrogen excretion, with 524 ± 53 and 471 ± 20 mg N/d/kg body weighted being excreted during the fasting state in the nonsupplemented and choline supplemented animals respectively.

Treadmill Study

The nonsupplemented and choline supplemented guinea pigs were run on a treadmill for either 2h or to exhaustion, whichever came first, to evaluate if the added stress of exercise would reveal differences in energy substrate utilization or endurance between the nonsupplemented and choline supplemented groups (**Figure 5-3**). The RER was not different between the nonsupplemented and choline supplemented groups.

Table 5-3
Urinary Excretion of Carnitine in Guinea Pigs With or Without Choline Supplement in Fed and Unfed States¹

Level of Choline Supplement and Feeding State	Carnitine (μmol/d)			
	NEC	ASAC	AIAC	TOTAL ²
Prior to Starting Experimental Diets				
(g/kg diet)				
0 - Fed	4.5 ± 0.7 ^a	2.8 ± 0.7 ^a	0.4 ± 0.1 ^a	8.0 ± 1.0 ^a
3 - Fed	4.6 ± 0.7 ^a	2.8 ± 0.8 ^a	0.5 ± 0.1 ^a	7.6 ± 1.4 ^a
0 - Unfed	1.5 ± 0.3 ^b	20.8 ± 2.9 ^b	0.8 ± 0.2 ^b	23.4 ± 3.1 ^b
3 - Unfed	1.6 ± 0.3 ^b	18.1 ± 2.0 ^b	1.1 ± 0.3 ^b	20.6 ± 2.1 ^b
While on Experimental Diets				
0 - Fed	4.8 ± 0.7 ^a	2.5 ± 0.5 ^a	0.4 ± 0.4 ^a	7.6 ± 1.1 ^a
3 - Fed	2.6 ± 0.7 ^a	1.5 ± 0.4 ^a	0.4 ± 0.1 ^a	4.5 ± 1.0 ^a
0 - Unfed	1.6 ± 0.2 ^b	21.7 ± 1.6 ^b	1.5 ± 0.5 ^b	24.7 ± 1.8 ^b
3 - Unfed	2.2 ± 0.3 ^b	19.5 ± 1.8 ^b	1.3 ± 0.4 ^b	23.0 ± 1.7 ^b

¹Non-supplemented animals were fed ground commercial guinea pig diet, choline supplemented animals were fed the same diet with 3 g/kg added choline from choline chloride. Food was withheld for 8h (for gas measurements) prior to placing the animals in the metabolic cages when unfed for 24h urine collections. When fed, the animals were given free access to food. Values are expressed as means ± SEM for the group (n=8 when animals were starved and n = 16 when fed). Different superscripts indicate significantly different means among groups (P < 0.05).

²NEC = non-esterified carnitine, ASAC = acid-soluble acyl carnitine, AIAC = acid-insoluble acyl carnitine, Total = total carnitine.

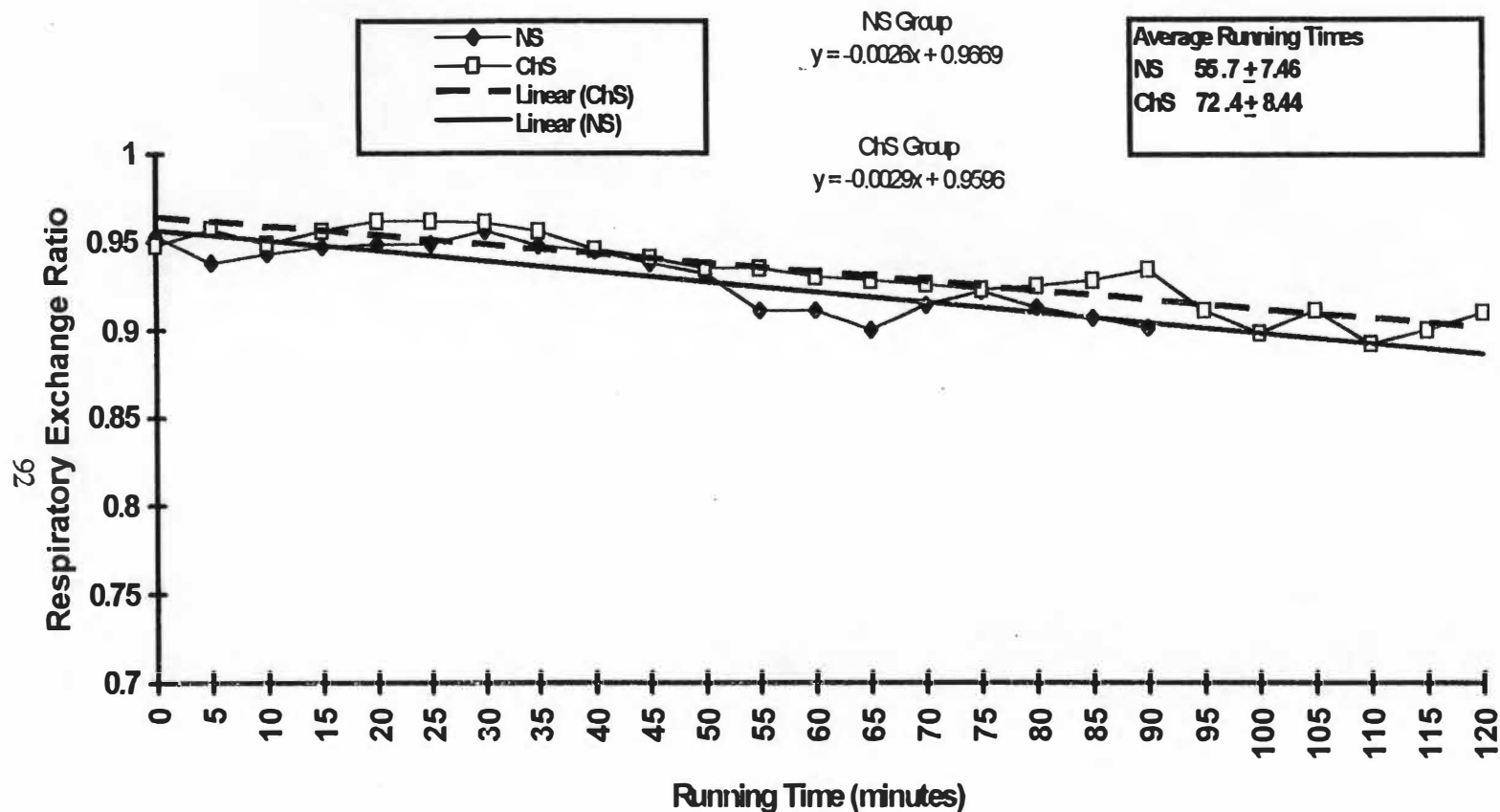


Figure 5-3: Respiratory exchange ratio of choline supplemented (ChS) or non-supplemented (NS) guinea pigs running on a treadmill at a speed of 15 m/min and an incline of 5%. The equation for the slope of each regression line is shown. Also shown is the average running time \pm SEM, there was no significant difference in running time by Student's t test ($P > 0.05$).

Final Blood, Urine, and Tissue Collections

After the animals had completed the starvation and treadmill studies, they were allowed to recuperate for two weeks, but remained on their respective diets. After recuperation, four animals (2 nonsupplemented and 2 choline supplemented) at a time were placed in metabolic cages and urine collected while fed or unfed (1 from each group for each treatment). After the 24h urine collection, blood and tissues were collected.

Urinary excretion of acyl and total carnitines was higher in starved animals, indicating increased oxidation of fatty acids. There was no differences in plasma total carnitine either between groups or as a result of starvation (**Table 5-4**). There was, however, a shift from mostly NEC to nearly equal amounts of NEC and ASAC in the plasma of starved animals. The excretion of carnitine was not different between the choline supplemented and nonsupplemented guinea pigs either during starvation or when fed (**Table 5-4**). Urinary excretion of acyl and total carnitine was significantly higher during starvation for both groups.

Brain total carnitine was higher in the choline supplemented group, due primarily to NEC, but was only significant in the unfed animals (**Table 5-5**). The unfed state tended to decrease liver free carnitine, but only significantly so in the nonsupplemented group. There was also a trend toward decreased carnitine in the hearts of unfed animals, due primarily to a decrease in ASAC. In kidney carnitine was higher in the NS animals during the fed state, but not different during starvation.

Table : 5-4
Final Plasma Concentrations and Urinary Excretions of Carnitine in
Control or Choline Supplemented Guinea Pigs in Fed or Unfed States¹

Level of Choline Supplement and Feeding State	Carnitine			
	NEC	ASAC	AIAC	TOTAL ²
(g/kg diet)	Urinary Excretion (μmol/d)			
0 - Fed	4.8 ± 1.01	2.1 ± 0.8 ^a	0.4 ± 0.1 ^{ab}	7.2 ± 1.4 ^a
3 - Fed	2.7 ± 1.14	1.5 ± 0.4 ^a	0.2 ± 0.1 ^a	4.4 ± 1.3 ^a
0 - Unfed	3.4 ± 0.6	13.8 ± 2.6 ^b	0.6 ± 0.1 ^{bc}	17.9 ± 3.3 ^b
3 - Unfed	4.3 ± 0.92	13.3 ± 2.9 ^b	0.7 ± 0.12 ^c	17.7 ± 2.6 ^b
	Plasma (μmol/L)			
0 - Fed	30.4 ± 3.5 ^a	9.0 ± 2.2 ^a	2.2 ± 0.3	41.6 ± 3.9
3 - Fed	31.3 ± 4.0 ^a	8.0 ± 2.1 ^a	2.0 ± 0.3	41.3 ± 5.1
0 - Unfed	20.5 ± 1.3 ^b	17.2 ± 1.6 ^b	2.0 ± 0.2	39.7 ± 1.0
3 - Unfed	20.7 ± 2.4 ^b	16.7 ± 2.5 ^b	1.8 ± 0.2	39.2 ± 4.7

¹Non-supplemented animals were fed commercial guinea pig diet, choline supplemented animals were the same diet with 3 g/kg added choline from choline chloride. Food was withheld from half of the animals (Unfed) in each dietary treatment for 24h prior to killing them. The remainder were given free access to food (Fed). All animals had completed the exercise study at least 2 weeks before being killed. Values are expressed as means ± SEM for the group (n = 5 for all groups except n = 4 for both fed groups for urine data and choline supplemented fed group for plasma data). Means among groups with different superscripts are significantly different (P < 0.05), by Student-Newman-Keuls test.

²NEC = non-esterified carnitine, ASAC = acid-soluble acyl carnitine, AIAC = acid-insoluble acyl carnitine, Total = total carnitine.

Table : 5-5
Tissue Concentrations of Carnitine in Guinea Pigs Fed Diets
With or Without Supplemental Choline in Fed or Unfed States¹

Level of Choline Supplement and Feeding State	Carnitine (nmol/gram wet tissue)			
	NEC	ASAC	AIAC	TOTAL
(g/kg diet)				
Brain				
0 - Fed	99.4 ± 22.5 ^a	16.3 ± 5.3 ^b	19.8 ± 1.5	135.5 ± 27.6 ^a
3 - Fed	120.3 ± 43.1 ^a	30.4 ± 14.8 ^{bc}	16.7 ± 5.1	167.4 ± 56.1 ^{ab}
0 - Unfed	71.1 ± 21.3 ^a	1.8 ± 0.8 ^a	20.2 ± 3.0	93.1 ± 24.4 ^a
3 - Unfed	196.6 ± 43.4 ^b	31.1 ± 5.4 ^c	18.2 ± 4.4	245.8 ± 39.5 ^b
Liver				
0 - Fed	338.6 ± 91.1 ^a	4.5 ± 2.6	13.3 ± 6.4 ^a	356.4 ± 91.6
3 - Fed	252.0 ± 44.6 ^{ab}	1.9 ± 1.9	31.5 ± 2.0 ^b	285.4 ± 43.9
0 - Unfed	186.6 ± 17.4 ^b	7.1 ± 4.9	22.9 ± 6.8 ^{ab}	216.6 ± 17.2
3 - Unfed	196.6 ± 43.4 ^{ab}	13.6 ± 6.8	26.7 ± 3.7 ^b	236.8 ± 47.0
Heart				
0 - Fed	654.5 ± 90.4	839.6 ± 84.6 ^a	134.1 ± 55.2	1628.2 ± 35.2 ^a
3 - Fed	744.6 ± 117.7	792.4 ± 208.3 ^{ab}	158.9 ± 101.1	1695.9 ± 259.9 ^{ab}
0 - Unfed	679.3 ± 154.9	600.3 ± 106.7 ^b	160.8 ± 15.6	1440.3 ± 139.8 ^{ab}
3 - Unfed	593.8 ± 96.4	563.5 ± 111.9 ^b	167.2 ± 61.6	1324.8 ± 76.7 ^b
Kidney				
0 - Fed	411.2 ± 30.3	76.8 ± 25.4 ^a	22.8 ± 9.2 ^{ab}	510.8 ± 11.9 ^b
3 - Fed	411.1 ± 17.7	28.6 ± 21.7 ^{ab}	14.0 ± 3.5 ^a	453.7 ± 18.2 ^a
0 - Unfed	478.4 ± 54.3	32.9 ± 14.7 ^a	22.5 ± 2.0 ^b	555.5 ± 47.8 ^b
3 - Unfed	457.8 ± 47.1	16.2 ± 8.5 ^b	24.5 ± 6.5 ^{ab}	498.5 ± 45.8 ^{ab}

Continued

Table 5-5 - Continued

Level of Choline Supplement and Feeding State	Carnitine (nmol/gram wet tissue)			
	NEC	ASAC	AIAC	TOTAL
(g/kg diet)				
Skeletal Muscle				
0 - Fed	454.8 \pm 70.9 ^{ab}	223.0 \pm 46.1	54.7 \pm 14.8 ^a	732.5 \pm 84.4
3 - Fed	632.3 \pm 117.8 ^b	272.7 \pm 69.4	57.9 \pm 10.5 ^a	962.9 \pm 163.8
0 - Unfed	377.0 \pm 56.2 ^a	208.4 \pm 77.8	127.8 \pm 17.2 ^b	713.1 \pm 79.6
3 - Unfed	389.6 \pm 91.9 ^a	320.2 \pm 85.9	129.3 \pm 32.0 ^b	839.1 \pm 108.7

¹NEC = non-esterified carnitine, ASAC = acid-soluble acyl carnitine, AIAC = acid-insoluble acyl carnitine, Total = total carnitine. Non-supplemented animals were fed ground commercial guinea pig diet, choline supplemented animals were the same diet with 3 g/kg added choline from choline chloride. Food was withheld from half of the animals (Unfed) in each dietary treatment for 24h prior to killing them. The remainder were given free access to food (Fed). All animals had completed the exercise study at least 2 weeks prior to being killed. Values are expressed as means \pm SEM for the group (n=5 except for all groups except n=4 the choline supplemented fed group). Means among groups with different superscripts are significantly different (P < 0.05), by Student-Newman-Keuls test.

There was a significant decrease in muscle NEC in both groups when starved. The muscle carnitine was not significantly different between NS and ChS animals when either fed or unfed.

Carnitine Palmitoyltransferase Studies

Starvation increased CPT activity in the nonsupplemented animals, but not in the choline supplemented animals. Malonyl-CoA inhibition was determined in all samples as an indication of how much of the activity was due to CPT-1 as opposed to CPT-II (Figure 5-4). The average I_{\max} for malonyl-CoA in the liver samples was 83%, indicating that most of the activity was due to CPT-1.

Discussion

There was one major findings in this study; there is not an increase in fatty acid oxidation in choline supplemented guinea pigs, that could be revealed by a lower RER. This finding may be the result of three factors. First, RER may not be the best functional consequence to evaluate, although the role of carnitine in fatty acid oxidation is the best characterized function of carnitine. Second, guinea pigs may not a be good model for studying fatty acid oxidation. Third, there may be no functional consequence of the choline induced increase in muscle carnitine concentrations.

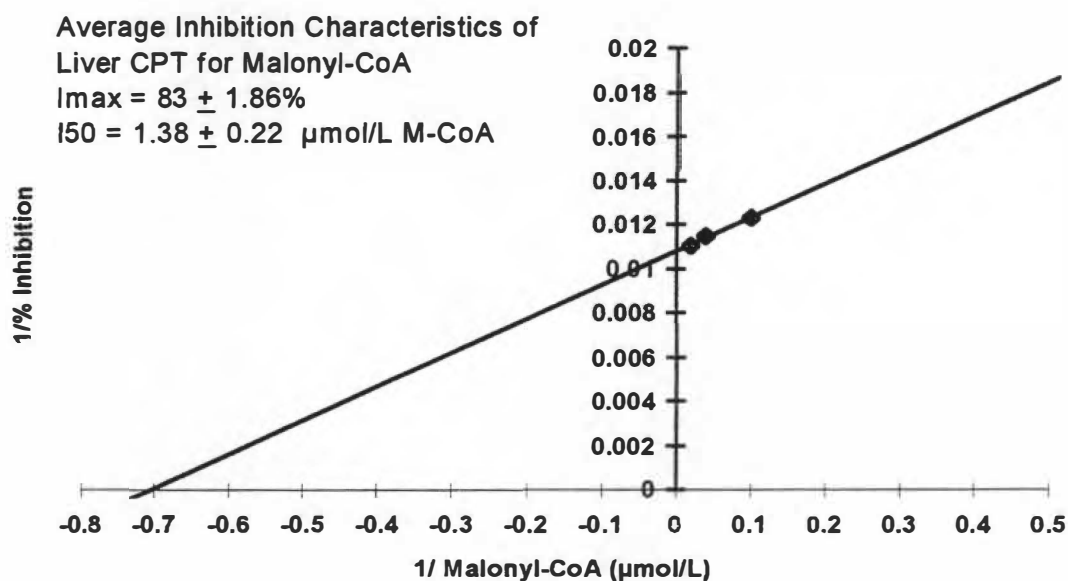
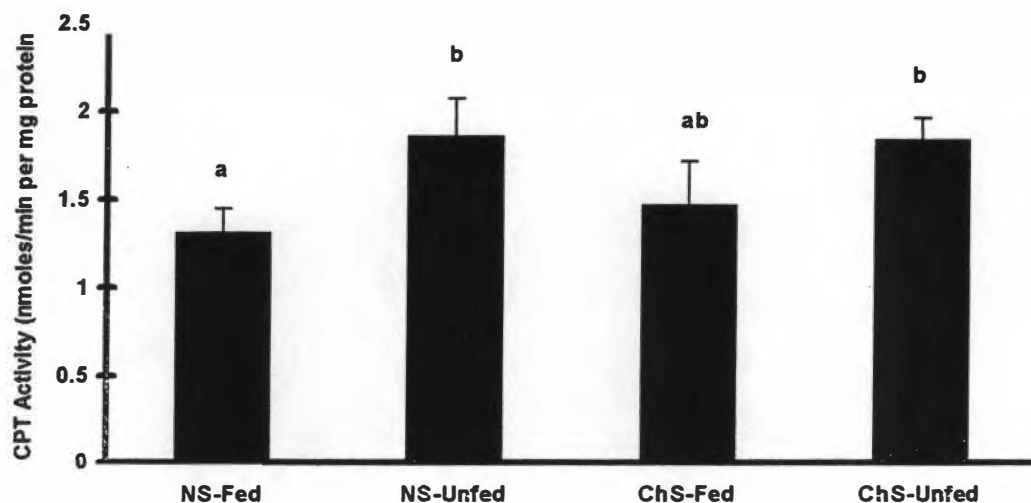


Figure 5-4: Activity of CPT (top) in liver of non-supplemented (NS) or choline-supplemented (ChS) guinea pigs fed or unfed for 24h. Values are means for the groups \pm SEM. Differences were evaluated by ANOVA and Student-Newman-Keuls test ($P \leq 0.05$, $n = 5$), different letters above bars indicate significant difference. Malonyl-CoA inhibition of liver CPT activity was evaluated using a double-reciprocal plot of malonyl-CoA inhibition as, explained in the materials and methods section. The curve shown here (bottom) is an example of 1 animal. Averages of I_{max} and I_{50} \pm SEM are shown.

RER as a Functional Consequence of Increased Tissue Carnitine

The failure of choline supplementation to effect the RER in guinea pigs, under any conditions, seems contradictory to the body composition data presented in chapter 4. Of course, previous studies on the effect of carnitine on athletic performance have been contradictory also (Table 5-1). There has been only one study that measured both muscle carnitine and RER under exercise conditions in carnitine supplemented people. That study found no effect of carnitine supplementation on either muscle carnitine or RER while exercising (Vukovich et al. 1994). However, carnitine supplementation did increase the muscle carnitine concentration in the study by Arenas et al (1991) but neither RER during exercise nor any other functional effect of carnitine was measured in that study. Other studies have found that carnitine does reduce RER (Gorostiaga et al. 1989, Wyss et al 1989), and one study found that work capacity was increased, without an effect on RER when carnitine was given 1h prior to exercise (Veechiet et al 1990). If tissue carnitine does increase work capacity, it seems logical to assume that it does so by increasing the capacity of the cell to transport fatty acids into the mitochondrial matrix for oxidation. However, carnitine availability for fatty acid transport may not be rate limiting. It must be remembered that carnitine functions to shuttle acyl moieties between cellular coenzyme-A pools. Removal of short-chain acyl groups from acyl -CoA in the mitochondrial matrix, thereby freeing Co-A, is an important function of carnitine and carnitine acetyltransferase (Bieber et al. 1992, Broquist 1994). The concentration

of coenzyme-A in human skeletal muscle is ≈ 10 nmol/g, whereas carnitine concentration is in the range of 2000 - 5000 nmol/g (Brass and Hiatt 1994). The intracellular carnitine pools is believed to act as a sink to help maintain an optimal free/acyl-CoA, which is important in the regulation of several important enzymes involved in intermediary metabolism, including pyruvate dehydrogenase (Bieber et al, 1992). It has been demonstrated that, during intense exercise, there is a shift to higher concentrations of muscle acyl-carnitine at the expense of free carnitine (Friolet, R., Hoppeler, H., and Krahenbuhl, S. 1994). There is a simultaneous increase in total coenzyme-A, and a significant increase in the ratio of acyl/free-CoA. The implications of changes in free and acyl CoA are difficult to interpret from measurements of total tissue concentrations. It is believed that during ketotic states, the intramitochondrial acyl/free-CoA ratio increases whereas the cytosolic acyl/free-CoA ratio decreases (Bremer, J. 1983, Siess et al, 1978). It is possible that the maintenance of free CoA can become the limiting action of carnitine during exercise, and that failure to do so may impair both fat and carbohydrate oxidation (Bieber et al, 1992, Sugden and Holness 1994).

Because this study evaluated the effect of choline on muscle carnitine, any effect that would affect neuromuscular signaling might also affect running ability. Carnitine in skeletal muscle may also have a role, through an interaction with choline, in the synthesis of acetylcholine. Acetylcholine is an important neurotransmitter in both nervous tissue and at the neuromuscular junction. Carnitine and acetylcarnitine

have been proposed to facilitate the production of acetylcholine in the brain, and carnitine acetyltransferase activity is known to be reduced in Alzheimer's disease (Kalaria, R.N. and Harik, S.H. 1992). In cultured neuroblastoma cells, the combination of choline and carnitine has been demonstrated to synergistically increase the production of acetylcholine (Wawrzenczyk et al 1994). Whether the combination of choline and carnitine in muscle cells could synergistically increase acetylcholine signaling at the neuromuscular junction has not yet been investigated, but this possibility can not be ignored as a possible functional consequence of the choline carnitine interaction investigated here, and deserves further investigation.

Guinea Pigs as a Model for Fatty Acid Oxidation

How efficiently guinea pigs utilize fat as an energy substrate has not been well documented. The CPT-I activity reported here for guinea pigs is only about 25-30% of that reported for rat liver, using the same assay (Kashfi et al 1993). The lower activity can not be due to contamination with CPT-II, since such contamination increases CPT activity under assay conditions. This is not surprising, since the reported K_m for carnitine in guinea pig liver CPT-I is $311 \mu\text{mol/L}$, as compared to $37 \mu\text{mol/L}$ and $39 \mu\text{mol/L}$ in rat liver and human fetal liver, respectively (McGarry et al. 1983). Guinea pig liver CPT-I is also more sensitive to malonyl-CoA inhibition than rat liver ($I_{50} = 1.38$ vs $2.7 \mu\text{mol/L}$). The high K_m for carnitine and the greater

sensitivity to malonyl-CoA both suggest that guinea pig may not be able to utilize fat for energy as efficiently as rats and humans.

The animal model may be responsible for the lack of effect on RER in this study, but it must be remembered that the studies in carnitine supplemented humans show conflicting effects on RER (Table 5-1). This may be due to the fact that there were very small differences in RER in the studies. For instance, 0.883 vs 0.904 was significant in the study by Wyss et al. (1990)). Nevertheless, repeating the exercise study in human subjects would result in better control, since running time can be better controlled in human subjects than in guinea pigs.

Tissue and plasma carnitine

Tissue carnitine concentrations tended not to be significantly different between groups, although there was a trend toward lower concentrations in starved animals which was sometimes significant. It may not be surprising that there was not a significantly higher muscle carnitine concentration in the choline supplemented, exercised animals since Arenas et al. (1991) demonstrated that exercise depletes the muscle of carnitine in humans. In this study plasma concentrations were the same between choline supplemented and nonsupplemented animals in contrast to higher plasma carnitine in previous choline supplemented animals. The lack of difference in plasma carnitine may again be due to an effect of exercise, which is similar to what has been seen in human studies.

Conclusions

The lack of effect of choline supplementation on RER suggests that any possible functional consequences of the choline mediated increases in tissue carnitine concentrations are independent of changes in fatty acid oxidation. Vecchiet et al. (1990) found that carnitine supplementation increased work capacity without increasing RER in humans, therefore suggesting a functional consequence of carnitine that is not related to the capacity for fatty acid oxidation. There is, therefore, the possibility of a functional consequence of the choline induced carnitine conservation that has not yet been investigated.

When feed was withheld from the animal there was an increase in fatty acid oxidation in the guinea pigs as evidenced by the decrease over 8 hours in RER and by the increased 24 h urinary excretion of acyl-carnitines. However, there was no effect of choline supplementation.

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Appendixes

Appendix 1

Carnitine Palmitoyltransferase Assay

Isolation of Mitochondria

1. Prepare mitochondrial isolation mixture (MIM) - 0.25 M sucrose with 1mM EGTA. To make 1 L dissolve 85.6g sucrose and 0.38 g EGTA in distilled water and adjust pH to 7.2 by adding 1M tris dropwise.
2. Remove liver from killed animal and place in a beaker with cold MIM, and chop into small pieces.
3. Swirl pieces of liver to suspend blood and small particles of connective tissue and decant. Repeat washing with MIM until blood is removed (2-3 times).
4. Homogenize liver using 3 passes of a Potter-Elvehjem homogenizer rotating at 125 rpm. Transfer to 1 or 2 50-ml centrifuge tubes
5. Centrifuge at 600 x g for 10 minutes.
6. Decant the supernatant containing the mitochondria into a second set of centrifuge tubes. Discard the pellet.
7. Centrifuge at 12,000 x g for 10 minutes.
8. Discard supernatant and resuspend the pellet in MIM using a teflon stirring rod to loosen the pellet, then transferring to a small Potter-Elvehjem homogenizer and resuspend gently by hand.
9. Recentrifuge at 12,000 x g for 10 minutes.
10. Discard the supernatant and resuspend the pellet, but bring to a total volume of only 3-4 ml this time.
11. Assay protein by the Biruet method and bring the mitochondrial suspension to 10mg protein/ml.

CPT Assay

Reagents and Solutions

1. Imidazole buffer (800 mmol/L): Dissolve 13.62 g imidazole in approximately 200 ml double-distilled water (DDW). Adjust pH to 7.12 at room temperature and bring to final volume of 250 ml in a volumetric flask.
2. Stock BSA (20%): Dissolve 10 g of bovine serum albumin (BSA) in approximately 40 ml DDW and bring to a final volume of 50 ml in a volumetric flask.
3. Antimycin A: Dissolve 10 mg in 10 ml ethanol.
4. Palmitoyl CoA (1mmol/L): Dissolve 10.42 mg of palmitoyl-CoA in 10 ml DDW.
Note: check purity of palmitoyl CoA to be certain of adding correct amount.
5. L-carnitine (50 mmol/L): Dissolve 98.85 mg of L-carnitine-HCl in 10 ml DDW.
Note: check purity of carnitine to be certain of adding correct amount.
6. [Methyl-3H]-L-carnitine: 250 μ Ci (1 mCi/ml) from Amersham.
7. Perchloric Acid (1 mol/L): Add 89.7 ml of 70% perchloric acid (PCA) to a half filled with DDW 1 L volumetric flask and bring to volume with DDW.
8. Saturated ammonium sulfate: Dissolve 100 g ammonium sulfate in 100 ml DDW and heat while stirring with a stir bar. Allow to cool and store in a bottle at room temperature.
9. Sucrose (2 mol/L): Dissolve 68.46 g sucrose in 50 ml DDW and bring to volume in a 100 ml volumetric flask.
10. Potassium Chloride (1 mol/L): Dissolve 7.64 g potassium chloride (KCl) in approximately 50 ml DDW and bring to volume in a 100 ml volumetric flask.
11. EGTA (0.1 mol/L): Dissolve 3.804 g EGTA in approximately 80 ml DDW, bring to pH 7.0 with 0.1 mol/L KOH. Bring to volume in a 100 ml volumetric flask.

12. Cocktail - I (250 ml)

Imidazole (800 mM)	87.5 ml
Sucrose (2M)	41.0 ml
EGTA (0.1 M).....	10.0 ml
Antimycin A (1mg/ml)	1.0 ml
BSA (20%).....	10.0 ml
KCl (1M).....	70.0ml

Adjust pH to 7.2 and bring to a volume 250 ml with distilled water.

13. Cocktail - II

L - Carnitine (50mM)	10.0 ml
[methyl ³ H] L-carnitine, 1 mCi/ml.....	0.4 ml

Bring to a total volume of 50 ml with distilled water.

Assay Procedure

1. Add to 16 x 100 mm test tubes - all samples should be run in duplicate
25 µl Cocktail II
Palmitoyl CoA According to protocol
Other Ingredients e.g. malonyl CoA
Distilled Water
2. Add enzyme (150 µg liver mitochondria, 75 µg heart mitochondria)
3. Incubate for 5 minutes @ 37°C in a shaking water bath.
4. Add 50 µl Cocktail II and incubate for 5 minutes. For this step the number of samples must be small enough to complete all within time with 5-10 second intervals between samples.
5. Stop reaction by adding 4 ml of 1N perchloric acid (PCA) and placing on ice.
6. Centrifuge for 10 minutes at 2000 rpm.
7. Decant and discard supernatant, add 0.4 ml 1N PCA and sonicate to dissolve pellet. Resuspend in 4 ml 1N PCA.
8. Centrifuge 5 minutes at 2000 rpm.
9. Decant and discard supernatant, add 0.4 ml 1N PCA, and sonicate.

10. Add 1.6 ml distilled water, 0.1 ml saturated ammonium sulfate, & 1.6 ml of n-butanol, and vortex.
11. Centrifuge for 10 minutes at 2000 rpm to separate aqueous and butanol phases.
12. Remove an aliquot (0.8 ml) of the upper (butanol) layer and transfer to a scintillation vial.
13. Add 5 ml scintillation fluid, mix and count.

Example of Assay Scheme - Running a Malonyl CoA Inhibition Curve

<u>Reagent (μl)</u>	Blank	Row A	Row B	Row C	Row D
Water	325	300	275	295	299
Cock I	125	125	125	125	125
Palmitoyl CoA	0	25	25	25	25
Malonyl CoA	0	0	25	5	1
Mitochondria	25	25	25	25	25
Cocktail II	25	25	25	25	25

Example of Tube Setup - 4 tissue sample labeled 1-4 in duplicate

		1D	1D	2D	2D	3D	3D	4D	4D
		1C	1C	2C	2C	3C	3C	4C	4C
		1B	1B	2B	2B	3B	3B	4B	4B
B	B	1A	1A	2A	2A	3A	3A	4A	4A

B = blank, letters following numbers indicate sample # with treatment from rows in the treatment scheme above.

Begin pipetting on the left with blanks and pipette Cocktail II into the tubes (with other ingredients already added) at 5 second intervals. When 5 minutes have lapsed since beginning with blanks begin adding PCA to the blank and pipette through the sequence at 5 second intervals.

Calculations

First calculate the specific activity of for carnitine in cocktail II:

Have: 50mM carnitine or 50 $\mu\text{mol/mL}$ \therefore have 50 nmol/ μL

Determine activity by scintillation counting in disintegrations per minute (DPM).

Example: 200,000 DPM for 10 μL of cocktail II (1/5 50mM carnitine)

10 μL of cocktail II has 2 μL of 50 mM carnitine or 100 nmol carnitine =

200,000 DPM/100 nmol carnitine or 2000 DPM/nmol of carnitine.

Calculate activity by the equation:

$$\text{Activity} = \left\{ \frac{DPM_{\text{sample}} - DPM_{\text{blank}}}{\text{SpecificActivity}(DPM / nmol)} \times \frac{1}{0.5} \right\} \div 5 \text{ min} \div \text{mg protein}$$

Activity is in nmol/minute/mg protein

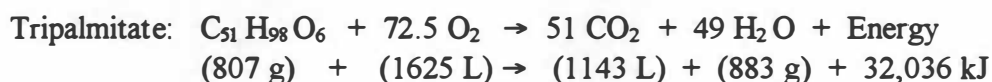
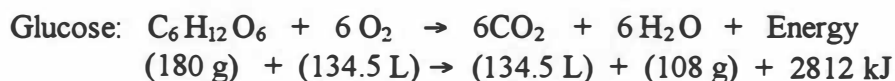
1/0.5 or 2 is a result of taking a 0.8 ml aliquot of the 1.6 ml butanol used for the extraction. 25 μL of 10mg/ml mitochondrial protein = .250 for mg protein.

Indirect Calorimetry - Methods

Metabolic Basis of Indirect Calorimetry

Because of the different oxidation states of fats, proteins, and carbohydrates; the oxidation of these nutrients result in different levels of O₂ utilization and CO₂ production. The following discussion is based on information from the book *Energy Metabolism, Indirect Calorimetry, and Nutrition* by Bursztein et al. (1989).

The stoichiometric equations for glucose and tripalmitate demonstrate the basis for indirect calorimetry for the determination of relative energy substrate use.



The respiratory quotient (RQ) of a person or animal can be used as an indicator of relative amounts of fat and carbohydrate oxidation. The RQ is obtained by dividing the CO₂ produced by the amount of O₂ utilized. Therefore, from the above equations the RQ for glucose and tripalmitate can be calculated as follows.

$$\text{Glucose: } 134.5\text{L CO}_2 \div 134.5\text{L O}_2 = 1.0$$

$$\text{Tripalmitate: } 1143.5\text{L CO}_2 \div 1625\text{L O}_2 = 0.703$$

Based on the RQ of expired gases, the relative amounts of fat vs carbohydrate can be estimated. Protein, however, contributes about 15% of energy needs during resting conditions (less during exercise) and has an RQ of about 0.81. Because of its midrange of RQ and small contribution to energy, protein is frequently ignored and RQ expressed as a “non-protein” R.

The term RQ for measurements in indirect calorimetry is commonly used in the literature, but it is technically incorrect. Oxidation of energy substrates occurs at the cellular level and RQ is the ratio of CO₂ produced and O₂ used at the cellular level, whereas the gases measured are those exchanged in the lungs. Gas exchange in the lungs is a good indicator of RQ only if body stores of O₂ and CO₂ do not change. What is actually measured is the respiratory exchange ratio (RER). Several factors can introduce differences between RQ and RER including:

1. Hyperventilation can result in excessive loss of CO₂ and a high RER (Fox et al. 1988).
2. Oxidation of fats result in a low RER, on the other had, excessive lipogenesis can result in a high RER, even when some fat is being oxidized, this is sometimes seen in patients receiving high carbohydrate TPN formulas (Bursztein et al. 1989).
3. Ketogenesis is a situation in which an O₂ poor substrate (fat) is converted into O₂ rich substrates (ketone bodies), which can result in a low RER. If the ketones are rapidly oxidized they have little effect on RER, but if levels become high enough that they are excreted into the urine, then RER may drop below 0.07 (Bursztein et al. 1989).
4. Gluconeogenesis, like ketogenesis is a process that converts O₂ poor substrates into O₂ rich substrates and thereby utilizing oxygen, increasing apparent O₂ utilization and lowering RER (Bursztein et al 1989).

Only rarely does the RQ or RER exceed 1.0 or drop below 0.7. The highest RER ever recorded was 1.4 which was in an over-fed goose. During periods of starvation the combination of gluconeogenesis and ketogenesis can cause RER to drop to about 0.68. It is rare, however, for RER to be outside the range of 0.7 - 1.0. It is usually assumed that at 0.7 the subject is relying almost entirely on fat for energy, at 1.0 almost entirely on carbohydrate, and at 0.85 the two contribute equally to energy needs (Bursztein et al. 1989).

Estimates of energy use are sometimes made from O_2 utilization alone. This is valid when the RER of subjects or groups being compared are similar, and results in only a small error when dissimilar. As can be seen from the equations for glucose and tripalmitate, glucose will yield 20.91 kJ of energy per L O_2 ($2812 \text{ kJ} \div 134.5 \text{ L } O_2$) and tripalmitate 19.71 kJ of energy per L O_2 ($32,036 \text{ kJ} \div 1625 \text{ L } O_2$). Comparing energy yield from O_2 utilization alone results in only about a 6% difference between 100% fat and 100% carbohydrate oxidation.

Gas Analysis - Equipment Setup

The gas analysis apparatus is setup as shown in **Figure A-1**. The usual procedure is to draw gasses through the O₂ and CO₂ sensors with the pump. The equipment used is:

- 1. Ametek N-22M** - oxygen sensor.
- 2. Ametek S-3A/II** - oxygen analyzer.
- 3. Ametek P-61B** - carbon dioxide sensor.
- 4. Ametek CD-3A** - carbon dioxide analyzer.
- 5. Ametek R-1** - flow control.

Caution must be observed to be certain that air lines are connected correctly.

When unreasonable measurements occur, the air lines should be checked to be certain. The equipment must be set up so that adequate ventilation is available to the instruments to prevent overheating. It is also important that the equipment be set up in an area with adequate ventilation so that the ambient gas concentrations will remain stable. Gas values from expired air are compared to ambient gas concentrations in the room (normally 20.93% and 0.03% for O₂ and CO₂ respectively). If the inspired room air changes from these values, erroneous values for gas utilization and production will result. The pump must be set to control the flow rate of gasses through the system. A flow rate of 50-300 ml/minute is acceptable, however 100

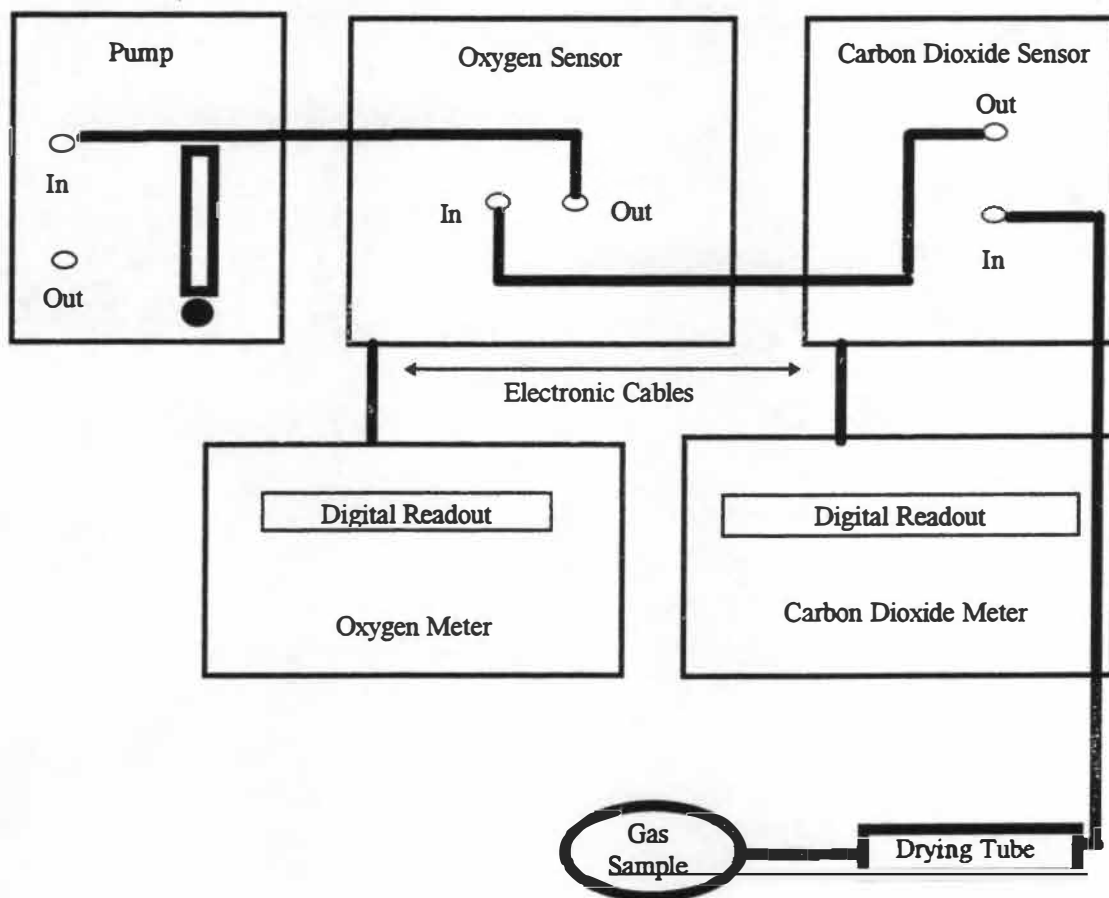


Figure A-1: Equipment setup for gas analysis. Gasses are pulled through the sensors by the pump. Heavy lines indicate gas lines except where labeled as electronic cables.

ml/minute is ideal for most applications. The flow rate is adjusted by turning the knob below the flow meter on the pump. A reading on the flow meter of approximately 1.25 mm corresponds to a flow rate of 100 ml/minute. For determining reading for other flow rates see Figure II-4 on page 28 of the manual for the Ametek oxygen analyzer.

Equipment Maintenance

Oxygen Analyzer

Approximately once a week the *cell restore cycle* should be activated. This is accomplished by using a pencil or small screwdriver to push the button inside the hole labeled “cell restore” on the front panel of the analyzer. This should only be done when the unit is running and gas flowing through the unit. Normally the unit is left running all the time, unless it is going to be unused for a long period of time. Gas lines need to be checked periodically to be certain that there are no leaks and that each is securely in place so that no contamination from outside air is getting into the system.

Caution must be observed to avoid allowing flammable gasses to pass through the system. Too much of a flammable gas could damage the analyzer when passed through the high temperature cell and ignited, small amounts would use oxygen for combustion and result in low oxygen values.

Carbon dioxide analyzer

As with the oxygen analyzer, the air lines need to be regularly inspected. Once a month the cells on the sensor need to be cleaned. To clean the cell first turn off the unit and allow to cool. Then disconnect the air lines, both in and out, and flush warm water with a detergent through the in and out ports - both directions. Then flush both directions with distilled water followed by flushing with alcohol. Flow clean dry air through the system, with the system still turned off, for at least one hour before using.

Note: I prefer to do both the cell restoration, and flushing operations on a Friday and allow the unit to run over the weekend, before using it for an experiment on Monday.

These procedures are the only routine maintenance for these instruments. For other trouble-shooting ideas and procedures, refer to the manuals.

Startup and Calibration

Drying tubes

Drying tubes need to be prepared for drying gasses before entering the analyses equipment. Tubes made from tygon tubing with connectors are prepared for this use. Prior to use, tubing is cleaned by removing old desiccant and placing new glass wool inside the connectors. One connector is placed on one side of the tygon tubing and the tube filled with desiccant from the other side. After filling, a connector is used to close the open end of the tubing. Air is blown through the newly filled drying tube to

remove dust, so as to avoid contamination of the instruments. One tube can be used for up to 4 hours.

Maintaining uniformly dry gasses is essential for accurate measurement of gasses, especially oxygen. It is possible to adjust measurements based on relative humidity, but drying gassed before measuring them is more convenient and the generally accepted practice. For a diagram of the effect of humidity on oxygen measurements see Figure II-3 on page 26 of the Ametek oxygen analyzer manual.

Oxygen Analyzer

1. Turn on the power at least 30 minutes prior to operation, 2 hours if possible. When using this instrument frequently, leave the power on all the time.
2. Set the dial to O₂%.
3. Set the reference/unknown switch to the reference position. Allow room air to flow through the system in the same manner as sample air will be. Set the cell zero potentiometer to midrange.
4. Turn the reference/unknown switch to unknown position and adjust the reference potentiometer until the display reads the correct O₂% (20.93% for room air). A single point calibration is adequate for the oxygen meter and room air is a good choice as a calibration gas for indirect calorimetry. See page 22 of the Ametek oxygen analyzer for more information on calibration gasses.

Carbon dioxide analyzer

1. Turn on power switch at the same time that the oxygen analyzer is turned on.
2. Flow room air through the system in the same manner that sample gas will be analyzed.
3. Adjust the zero potentiometer until the display reads 0.03% (the CO₂% of room air), if using room air.
4. Flow a calibration gas with approximately 5% CO₂ through the system, for which the exact concentration is known, and adjust the span potentiometer until the display reads the correct concentration.

Whenever flowing gasses through the system it is imperative that the gasses not be forced through under pressure. This may result in a rupture of the cells and expensive repairs! Gas from a tank may be sampled by placing a small tube from the gas analyzer inside a much larger tube from the tank. The large tube must be large enough to allow free escape of gas from around the small tube, thereby preventing pressure from the tank from forcing gasses through the system. The system can also be checked by burning methanol in an alcohol burner. Methanol burns at an RQ of 0.67 and this can be used to determine if the system is working well. The alcohol must be burned at a very low flame to avoid overheating the chamber and possibly setting it on fire. Calibration gasses can also be purchased, these work well,

but the alcohol burner can be placed in the cage and test the whole system as it will be used with an animal.

Note: These instructions are basic reminders of routine procedures. Before using the equipment for the first time, the manuals should be studied.

Gas Sampling

The most important consideration for gas sampling is that a thoroughly mixed, representative sample be collected. The change in gas concentration from ambient air to that exiting in the chamber housing the animal being studied, times the air flow rate is the amount of oxygen utilized and carbon dioxide produced. The basic equations are:

$$VO_2 = (VI \times FIO_2) - (VE \times FEO_2)$$

Where: VO_2 = volume of oxygen utilized.

FIO_2 = Fraction of oxygen entering the chamber (0.2093 for room air).

FEO_2 = Fraction of oxygen exiting the chamber.

VE and VI = the air flow in and out of the chamber and are assumed to be equal.

$$VCO_2 = (VE \times FECO_2) - (VI \times FICO_2)$$

Where: VCO_2 = volume of carbon dioxide produced.

$FECO_2$ = fraction of carbon dioxide exiting the chamber.

$FICO_2$ = fraction of carbon dioxide entering the chamber (0.0003 for room air).

VE and VI = air flow in and out of the chamber and are assumed to be equal.

See material and methods for more details on calculations.

Both systems used in this dissertation research used fans to mix the gasses. This prevents air currents forming in the chamber and sampling of gasses that are not representative of the gas content of the chamber.

Air Flow Rates:

The concentration of gasses in the cage are affected by several factors, including:

- 1) the size of the chamber, 2) the size of the animal, and 3) the flow rate of air through the chamber. Greater changes in the gas concentrations have the advantage of making small drifts in calibrations of less consequence. On the other hand, too great CO_2 and too low O_2 concentrations will introduce metabolic effects that are not intended. To avoid hypoxic conditions the CO_2 concentration should not exceed 1%. In general a air flow rate of 2-3 L/minute will give the desired results.

Setting up the dessicator chamber:

The plastic dessicator chamber is used to measure gasses in resting animal. An air line is run from the oil free vacuum/pressure pump to the air flow valve and then the glass flow meter. From the glass flow meter the air line goes to the side tube on the cage. The air line from the top of the cage takes the air out of the cage to a second flow meter which monitors the air leaving the cage to insure that adequate air is flowing through the chamber and that there are no major air leaks. A small loss of air flow is not a problem, as long as there is a positive pressure of air so that air is not

leaking into the chamber from the outside. The flow rate going into the chamber is used for calculations and only a small percentage of the air coming out of the chamber is used for analysis. The air coming from the second air flow meter is collected into a gas collection bag for analysis.

Steps in using the dessicator chamber and treadmill:

1. Place a new drying tube on the air line going to the gas analysis instruments.
2. Calibrate the instrument after allowing dry air to flow through the instruments for at least 5 minutes.
3. Check the gaskets on the end covers of the treadmill unit, or place gasket grease on the surfaces of the dessicator unit.
4. Connect the hose from the flow meter attached to the air pump to the air inlet at the top of the end plate behind the fan on the treadmill, or to the tube at the side of the dessicator unit.
5. Place an animal in the treadmill chamber or dessicator unit and seal. Adjust the gas flow rate - a lower flow rate is frequently used for the resting baseline than for exercising animals.
6. Turn on the air pump and electrical shock coils on the treadmill, check the air flow meters to make certain that adequate air is flowing through the chamber.
7. Allow gasses to equilibrate for 10 minutes before collecting gasses.

8. Collect gas by inserting hose on gas collection bag into the hole at the bottom of the end plate behind the fan of the treadmill from the line from the out air flow meter.
10. Turn on treadmill while measuring gasses from non-exercising baseline collection.
12. Close the gas bag and mix gasses in the bag by pressing and releasing the bag.
13. Attach the bag to the drying tube leading to the analysis instruments.
14. Allow instrument readings to stabilize for 30 seconds and record the values.
15. Remove the gas bag from the drying tube and allow the instruments to re-calibrate to room air. Do not be too quick to adjust the instruments, the oxygen analyzer takes a long time to recover to the baseline readings. The CO₂ readings will change more quickly than O₂ since the gas passes through the CO₂ sensor first.
16. Remove air from the gas collection bag by attaching it to the vacuum side of a vacuum pump.

Vita

James William Daily III was born on July 10, 1948 in Chattanooga, Tennessee. He spent most of his early life in the town of Rockwell, North Carolina where his father owned a factory manufacturing stereo speaker systems. He attended high school at Mount Pisgah Academy, in Canton, N.C. and graduated in May, 1966. Following high school he attended Southern College of Seventh-Day-Adventists, and graduated with a Bachelor of Arts degree in History in May, 1970.

Following graduation he worked at various jobs including managing his father's factory, teaching mountain climbing, and as a real estate agent. During this time he married Ruth E. Gust and had three children, Anne, Jackie, and Jimmy.

In 1980 he opened Eden Way Natural Foods in Knoxville, Tennessee which he owned and operated until 1991. While operating his store, he recognized a need for a better understanding of the scientific aspects of nutrition and began taking classes at the University of Tennessee. His quest for a knowledge of nutrition led to his eventual enrollment as a graduate student, and ultimately this dissertation.